Nonerythrocyte Spectrins: Actin-membrane Attachment Proteins Occurring in Many Cell Types

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ABSTRACT The properties of brain fodrin have been analyzed and compared with those of erythrocyte spectrin. Both proteins consist of high molecular weight polypeptide doublets on SDS polyacrylamide gels and in solution behave as very large asymmetric molecules. Both proteins show a characteristic increase in sedimentation coefficient in the presence of 20 mM KCl. Antibodies against the brain protein cross-react with erythrocyte spectrin and cross-react with similar high molecular weight doublet polypeptides in SDS polyacrylamide gels of other cell types and plasma membrane preparations. Both proteins bind actin. The brain protein and erythrocyte spectrin show specific and competitive binding to erythrocyte membranes and this binding is inhibited by antibodies against erythrocyte ankyrin. Several of these properties distinguish these proteins from the class of high molecular weight actin-binding proteins that includes filamin and macrophage actin-binding protein. We conclude that together with erythrocyte spectrin, the brain protein and equivalent, immunologically related proteins in other cell types belong to a single class of proteins with the common function of attachment of actin to plasma membranes. Based on the structural and functional similarities, the name spectrin would seem appropriate for this whole class of proteins.

A major, unresolved problem in understanding cell movement is the question of how elements such as actin and myosin transmit force to the plasma membrane. Although there are many examples in which actin filaments have been demonstrated to be associated with membranes, only in the erythrocyte membrane are the molecular details of this attachment known (5). Initially, when the role of spectrin was being elucidated in erythrocytes, it was generally thought that spectrin might be involved in actin-membrane attachment in other cells as well. Immunological evidence, however, was presented against the existence of spectrin in other cell types (20) and thoughts on the universality of the spectrin-actin system were generally abandoned.

While investigating the role of α-actinin in the association of actin with isolated Hela cell plasma membranes several years ago (10), we noted a high molecular weight polypeptide doublet on SDS gels that was reminiscent of spectrin, being eluted from these membranes under low ionic strength conditions which also eluted actin. The Hela plasma membranes were insufficient as a source for purifying this protein, although we could show that it bound actin and was distinct from filamin (Burridge, unpublished results). Subsequently, finding that brain was very rich in this or a similar protein we developed a purification for it. While we were involved in the characterization of this protein from brain, Levine and Willard (23) described the isolation and cellular localization of the same protein, which they have named Fodrin.

In this paper we compare brain fodrin with spectrin from erythrocytes. Not only do these two proteins share a number of physical and structural properties, but they show immunological cross-reactivity and, most significantly, reveal functional similarities. These findings lead us to conclude that erythrocyte spectrin, brain fodrin, and its immunologically related equivalents in other cell types all belong to a family of related proteins - the spectrins. Because these proteins are related both physically and functionally, we prefer the name brain spectrin to fodrin. It seems likely that this class of proteins, the spectrins, functions in many higher cell types to mediate actin attachment to the plasma membrane.

MATERIALS AND METHODS

Purification of Brain Spectrin (Fodrin)

Frozen beef or pig brains were used, the former being more available at Cold Spring Harbor Laboratory where the work was initiated by one of us and where the antibody used in this study was raised. At the University of North Carolina, Chapel Hill, because of availability, pig brains have been used as the source of

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protein. No differences between the proteins from these two sources have been detected.

Frozen brains were chopped with a carving knife and then homogenized in a Waring blender (Dynamics Corp. of America, New Hartford, CT) at a medium setting with 8 vol of deionized water (4°C) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was centrifuged at 12,000 rpm for 10 min at 4°C in a Sorvall GSA rotor (DuPont Instruments, Newtown, CT). The supernatant was discarded. The pellet was resuspended in 8 vol of Buffer A at 37°C (2 mM Tris, 1 mM EGTA, 0.5 mM PMSF, pH 9.0 at room temperature). The suspension was stirred gently at 37°C for 30 min. The centrifugation was repeated and the supernatant saved. The pellet was reextracted with 8 vol of Buffer A at 4°C for 30 min. The centrifugation was repeated and the supernatant combined with the previous supernatant. These were fractionated with ammonium sulfate. Initially, 14.9 g of $(NH_4)_2SO_4$ were added per 100 ml of supernatant. After stirring for at least 1 h at 4°C the precipitated protein was removed by centrifugation. To the supernatant 11.2 g of $(NH_4)_2SO_4$ were added per 100 ml. After stirring for at least 1 h at 4°C the precipitated protein was collected by centrifugation (10 min at 10,000 rpm in a GSA rotor). The pellet was resuspended in 1 mM EDTA, 1 mM Tris, 0.1% β-mercaptoethanol, pH 7.6 and dialyzed overnight against this buffer. After dialysis the protein solution was centrifuged for 1 h at 40,000 rpm in a Beckman Ti 42.1 rotor (Beckman Instruments, Inc., Fullerton, CA). The resulting pellet was usually large and was reextracted in several volumes of the above buffer. The centrifugation was repeated and the supernatants from both centrifugations pooled and passed over a DEAE-cellulose (Whatman DE52) ion exchange column, equilibrated in Buffer B (20 mM NaCl, 0.1 mM EDTA, 0.1% β-mercaptoethanol, 20 mM Tris-acetate, pH 7.6). The protein was eluted with a salt gradient going from Buffer B to Buffer B plus 0.6 M NaCl. The brain spectrin eluted as a broad, trailing peak in the second half of the gradient. Fractions rich in brain spectrin as judged by SDS polyacrylamide gel analysis were pooled and concentrated by precipitation at 50% saturated $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation and resuspended in a small volume of Buffer B. This was loaded onto a gel filtration column (90 cm x 2.5-cm diameter) of Sepharose Cl-2B (in early experiments) or more recently, onto a Sepharose 4B column. Again the eluting fractions were monitored by gel electrophoresis. The peak brain spectrin fractions were pooled and passed over a column of hydroxyapatite (Bio-Rad Laboratories, Richmond, CA) equilibrated in 20 mM potassium phosphate, 0.1% β-mercaptoethanol, pH 7.5, and the brain spectrin was eluted with a potassium phosphate gradient, from 20–400 mM containing 0.1% β-mercaptoethanol at pH 7.5. The fractions containing brain spectrin were pooled and dialyzed into Buffer B in which the brain spectrin could be stored at 4°C for several weeks with little indication of proteolytic breakdown. The brain spectrin from the hydroxyapatite column was used in most of the experiments described below but for some preparations an additional chromatography step on phosphocellulose (Cellex-P, Bio-Rad Laboratories), was performed either before or after the hydroxyapatite chromatography. The phosphocellulose was equilibrated in Buffer B and eluted with a gradient from Buffer B to Buffer B containing 0.5 M NaCl.

All preparations of brain spectrin contained a contaminating lower molecular weight doublet on SDS polyacrylamide gels (~160,000 and 150,000 mol wt). Several lines of evidence lead us to conclude that these are proteolytic degradation products. Their ratio to brain spectrin varies in different preparations. They cross-react immunologically with antibodies against brain spectrin, but are not detected in gels of whole cells or tissues (Fig. 1). Partial digestion of the native brain spectrin with Staphylococcal V8 protease generates a similar polypeptide doublet (see Fig. 9). In one preparation in which chromatography was performed on hydroxyapatite before Sepharose gel filtration, the amount of this lower molecular weight doublet increased considerably during the hydroxyapatite step. Since this was the only stage of the preparation where calcium was present in the absence of calcium chelators, we suspect that this lower molecular weight doublet is generated by a calcium-activated protease that is not inhibited by PMSF.

Immunological Methods

Antibodies against the purified beef brain spectrin were raised in rabbits. Injections were given at about 3-wk intervals, the first being of the native protein in Freund's complete adjuvant and the next two being of the protein eluted from preparative SDS polyacrylamide gels. The second injection was also in Freund's complete adjuvant, the third was in incomplete adjuvant.

Antibodies against the 72,000-dalton proteolytic fragment of human erythrocyte ankyrin were raised in rabbits using a similar schedule of immunization. The 72,000-dalton fragment was purified following the procedure of Bennett (1). For immunization the protein was eluted from preparative 7.5% SDS polyacrylamide gels.

Immuno-autoradiography on SDS polyacrylamide gels was performed as described previously (7, 8) with whole serum diluted 1:30. Indirect immunofluorescence was performed essentially as described previously (9) using a Leitz Orthoplan microscope. Rabbit antibrain spectrin was used at a dilution of 1:50 or 1:100. Mouse monoclonal antibody against actin was the generous gift of Dr. J. Lin (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and was used at a dilution of 1:500. Fluorescein-labeled goat anti-rabbit and rhodamine-labeled goat anti-mouse antibodies from Cappel Laboratories (Cochranville, PA) were used at 1:50 or 1:100 dilutions.

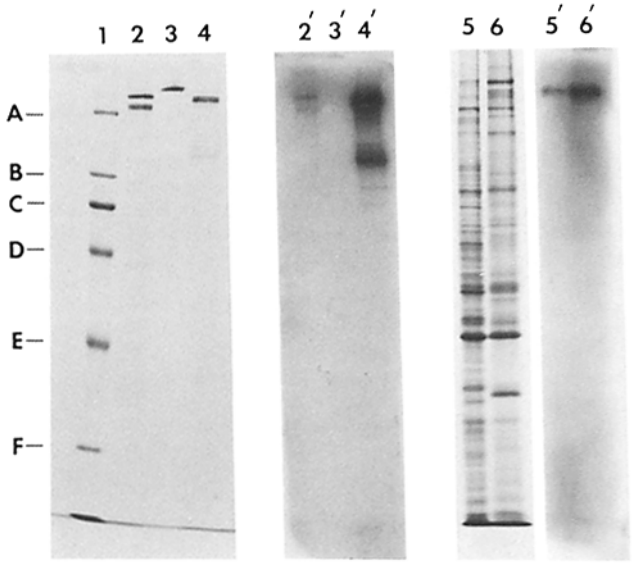

FIGURE 1 SDS polyacrylamide gel analysis of brain spectrin. Lanes *1–4* show a photograph of part of a gel stained with Coomassie Blue. Lane *1* contained molecular weight markers (*A*, myosin = 200,000; *B*, β-galactosidase = 130,000; *C*, phosphorylase = 95,000; *D*, BSA = 68,000; *E*, ovalbumin = 42,000; and *F*, carbonic anhydrase = 30,000). Lane *2* contained erythrocyte spectrin, lane *3*, filamin, and lane *4*, pig brain spectrin (fodrin). Lanes *2′–4′* show an autoradiograph of a parallel gel slice corresponding to lanes *2–4* that was first stained with rabbit anti-brain spectrin followed by ^{125}I-labeled goat anti-rabbit IgG. Note the labeling of erythrocyte spectrin (lane *2′*) and lack of labeling of filamin (lane *3′*). Lanes *5* and *6* show a photograph of a gel slice on which whole chick embryo fibroblasts (lane *5*) and crude Hela cell plasma membranes (lane *6*) were electrophoresed. A corresponding autoradiograph is shown (*5′*, *6′*) after a parallel gel slice was stained with anti-brain spectrin followed by immuno-autoradiography. Note the high molecular weight cross-reactive material, which on some occasions could be resolved as a tight doublet of bands.

Spectrin Binding to Erythrocyte Membranes

Inverted erythrocyte vesicles depleted in spectrin and actin were purified from human erythrocytes by the procedure of Bennett and Branton (3), except sedimentation on a T100 dextran gradient was omitted and a second extraction of spectrin at room temperature for 20 min was added.

Spectrin was purified from human erythrocytes by the procedure of Bennett and Branton (3). Erythrocyte and brain spectrins were concentrated by dialysis against 70% sucrose (wt/vol) in binding buffer (see below) followed by exhaustive dialysis against binding buffer. For binding studies erythrocyte and brain spectrins were radioiodinated by the chloramine-T method (21) using iodine-125 (New England Nuclear, Boston, MA). To the proteins in 50–100 μl of 50 mM potassium phosphate, pH 7.5, ~1 mCi of ^{125}I was added in 30 μl of the same buffer, followed by 5 μl of chloramine T (5 mg/ml) freshly prepared in deionized water. After ~2 min at room temperature the iodination was stopped by adding 10 μl of phosphate buffer saturated with L-tyrosine. After 5 min the iodinated proteins were separated from free iodine and iodotyrosine by gel filtration on Sephadex G-50, equilibrated in 20 mM KCl, 2 mM potassium phosphate, pH 7.6, 0.1% β-mercaptoethanol, and 0.2 mg/ml gelatin. The iodinated proteins were stored in this buffer with 8 mg/ml bovine serum albumin (BSA) at 0–4°C.

Binding studies were performed using the procedure of Bennett and Branton (3). Inverted vesicles were used at a concentration of 83 μg/ml of membrane protein except where indicated. Radioiodinated spectrins were diluted 1:10 to 1:30 in binding buffer (2.5 mM potassium phosphate, pH 7.6, 20 mM KCl, 1 mM $MgCl_2$, 0.1% β-mercaptoethanol) containing 1% BSA and centrifuged at 18,000 *g* for 40 min before use to remove any aggregated material. ^{125}I-erythrocyte

spectrin was used at a concentration of 0.3–1.0 μg/ml (sp act 3.67×10^6 cpm/μg; approximately one iodine atom per spectrin dimer). ^{125}I-brain spectrin was used at a concentration of 2 μg/ml (sp act, 1.35×10^6 cpm/μg). Radioactivity was measured by liquid scintillation counting in Aquasol (New England Nuclear). Controls were performed using heat-denatured brain or erythrocyte spectrin (10 min at 55°C). Samples were incubated for 90 min at 0–4°C in a final volume of 50 μl in binding buffer containing 1% BSA to prevent nonspecific binding. Then 40 μl was layered onto a cushion of 350 μl of 20% sucrose in 0.7 mM sodium phosphate, pH 7.6, 20 mM KCl, 1% BSA in a 0.5 ml microfuge tube and centrifuged for 30 min at 18,000 *g*. The microfuge tubes were frozen in liquid nitrogen, the tips cut off and counted for radioactivity. All assays were done in duplicate.

Binding studies in the presence of antibodies against ankyrin used crude immunoglobulin fractions prepared from preimmune or immune sera that were precipitated once with 45% saturated ammonium sulfate. The precipitated immunoglobulin fraction was resuspended and dialyzed against PBS. Before the experiments, 300 μl of these immunoglobulin fractions were chromatographed on 10 cm x 0.7 cm Sephadex G-50 columns, equilibrated with binding buffer. 30 μl of the peak fractions eluting from these columns was used in a preincubation step with 10 μl of the inverted vesicle preparations. After 30 min at 0–4°C, 10 μl of iodinated brain or erythrocyte spectrin was added and the binding assay was performed as usual.

Cell Culture

Gerbil fibroma cells (CCL 146) and mouse C3H 10T½ fibroblasts were cultured in Dulbecco's modified Eagle's medium (DME) containing 2 or 10% fetal calf serum (FCS), respectively. Primary cultures of beef adrenal medulla cells were the generous gift of Dr. N. Kirshner (Duke University, Durham, NC) and were cultured in a medium containing 45% DME, 45% nutrient mixture F12 (Ham) and 10% FCS.

Other Methods

Sedimentation coefficients were determined by the procedure of Martin and Ames (26) using sedimentation on 5–20% sucrose gradients containing either 0.3 mM sodium phosphate, pH 7.6, 0.01% β-mercaptoethanol, or this same buffer containing 20 mM KCl. Proteins were dialyzed overnight into the buffers corresponding to the sucrose gradients. Standard proteins and their respective S values included BSA (4.6), α-actinin (6.4), filamin (9.0), and catalase (11.2). Gel filtration was performed on a 90 × 2.5-cm column column of Sepharose CL-2B equilibrated in Buffer B with proteins equilibrated in the same buffer.

Proteolytic digestions of erythrocyte and brain spectrin were performed using both proteins at 0.4 mg/ml and Staphylococcal V8 protease or trypsin at a weight ratio of 1:200. Digestions were performed in Buffer B (above) at 21°C and were stopped at successive time points by withdrawing aliquots and immediately boiling in SDS gel sample buffer. Digestions were analyzed on 10% SDS polyacrylamide gels.

SDS polyacrylamide gels were run according to the procedure of Laemmli (22). 10% polyacrylamide gels contained 0.13% bisacrylamide. Protein concentrations were determined by the method of Lowry (24) or by the absorbance. Erythrocyte spectrin concentrations were determined by absorbance at 280 nm assuming an $A_{280}^{1\%}$ of 10.1 (11). Because of its structural similarities the same assumption was made for the purified brain spectrin. For actin an $A_{290}^{1\%}$ of 6.38 was used for G-actin and 6.17 for F-actin (17). Apparent viscosity was determined using a low-shear falling ball viscometer as described by MacLean-Fletcher and Pollard (25).

Plasma membranes were isolated from Hela cells grown in suspension using the procedure of Brunette and Till (6). α-Actinin, vinculin, and filamin were purified as previously described (13).

RESULTS

Physical Properties of Brain Spectrin (Fodrin)

The purified brain spectrin is shown electrophoresed on a 10% SDS polyacrylamide gel (Fig. 1, lane *4*) where it is compared with other high molecular weight actin-binding proteins, myosin, erythrocyte spectrin, and filamin. As described by Levine and Willard (23) the brain spectrin (fodrin) migrates as a tight doublet of bands. These migrate close to the α-subunit of erythrocyte spectrin (240,000 mol wt) and we have estimated the molecular weights of the two polypeptides as 240,000 and 235,000.

In solution, the brain spectrin behaves as a large asymmetric protein eluting on Sepharose CL-2B gel filtration columns with a K_{av} of 0.46, compared with erythrocyte spectrin dimers which have a K_{av} of 0.58. Such analysis indicates a Stokes' radius >200 Å for the brain protein. The sedimentation coefficient for the brain spectrin is 10S in 0.3 mM sodium phosphate, pH 7.6 but increases to at least 12S when 20 mM KCl is included in the buffer (higher S value aggregates are also evident) (Fig. 2). A similar shift in S value is seen for erythrocyte spectrin, from 8S to 10.4S in 20 mM KCl as has been noted previously (3, 11). The standard proteins show very little change in S value, except for filamin which shows less of an apparent increase than the brain or erythrocyte spectrins (Fig. 2). The increase in S value for the two spectrins is fully reversible upon dialysis back to the low ionic strength conditions.

Immunological Cross-reaction of Brain Spectrin with Erythrocyte Spectrin

Antibodies against the purified brain protein have been used to stain gels of pure proteins and crude cell fractions (Fig. 1). The antibody against the brain protein binds to erythrocyte spectrin, but does not label smooth muscle filamin (Fig. 1). The cross-reaction with erythrocyte spectrin is, however, weak and only seen when the parallel lane containing the brain protein is overexposed by comparison. A prominent doublet at

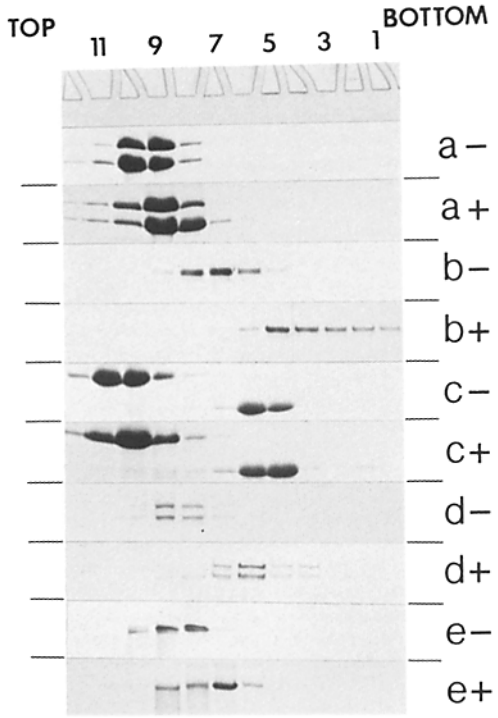

FIGURE 2 SDS gel analysis of proteins sedimented on sucrose density gradients. Sections of SDS polyacrylamide gels are shown on which fractions from the sucrose gradients were analyzed. Linear gradients of 5–20% sucrose without (−) or with (+) 20 mM KCl were centrifuged at 4°C for 15 h at 40,000 rpm in an SW41 Ti rotor (Beckman Instruments). Twelve equal-volume fractions were collected for each gradient, starting from the bottom. For easy comparison, only small sections of the gels are shown. *a* shows the gradients for vinculin and α-actinin, *b* shows the gradients for brain spectrin, *c* shows the gradients for BSA and catalase; *d* shows the gradients for erythrocyte spectrin; and *e* shows the gradients for filamin. Note how brain (b+) and erythrocyte (d+) spectrins sediment in the presence of 20 mM KCl to lower positions in the gradients.

~160,000 and 150,000 mol wt is also labeled by the antibody in the gel of the brain protein. We believe these bands correspond to proteolytic break down products of the high molecular weight polypeptides (see Materials and Methods).

The same antibodies label a high molecular weight polypeptide doublet in gels of whole cell lysates, for example in gels of chick embryo fibroblasts (Fig. 1). Crude plasma membrane preparations of Hela cells also display a high molecular weight

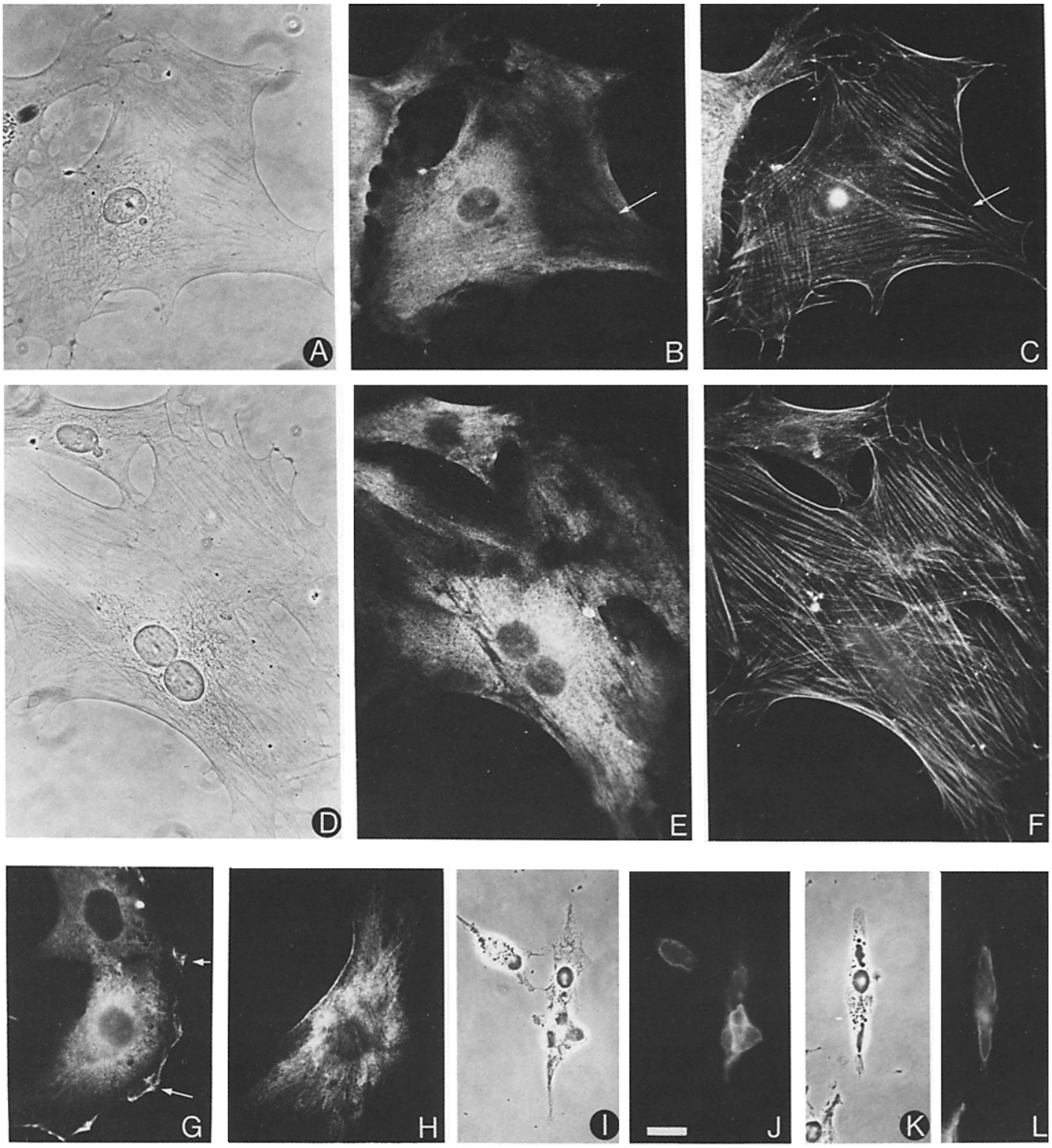

FIGURE 3 Immunofluorescent localization of spectrin in various cell types. *A–F* show gerbil fibroma cells stained with rabbit anti-brain spectrin and a mouse monoclonal antibody against actin. The antibodies were revealed with fluorescein-labeled goat anti-rabbit and rhodamine-labeled goat anti-mouse immunoglobulin respectively. *A* and *D* show phase micrographs, *B* and *E* show the distribution of spectrin visualized with fluorescein-specific optics, *C* and *F* show the distribution of actin visualized with rhodamine-specific optics. Note the lack of correlation between the actin and spectrin distributions. The arrows in *B* and *C* marks an actin microfilament bundle that corresponds to an absence or decreased staining of spectrin. *G* and *H* show immunofluorescent micrographs of mouse fibroblasts (10T½ cells) stained with anti-brain spectrin. In *G*, the cells were spreading after replating, and the ruffling membranes (arrows) show prominent spectrin localization. *I–L* show primary cultures of beef adrenal medulla cells stained with the anti-brain spectrin. *I* and *K* are phase micrographs, *J* and *L* are the corresponding immunofluorescent micrographs, but photographed at a slightly higher plane of focus. Note the peripheral localization of spectrin at the margin of these cells. All micrographs were taken at the same magnification. Bar, 16 μm.

immunologically cross-reactive polypeptide doublet that is distinct from filamin (Fig. 1).

Immunofluorescent Localization of Brain Spectrin in Different Cell Types

The antibodies against brain spectrin have been used to localize the protein in different cell types (Fig. 3). In some cells the distribution has been compared with that of actin by double-label immunofluorescence using a mouse monoclonal antibody against actin. In cultured fibroblasts the distribution is similar to that described by Levine and Willard (23) showing a diffuse, mottled distribution over the whole cell, unlike the distribution of any other known actin-binding proteins. This pattern was not seen with preimmune sera and was abolished by adsorption with the purified antigen (data not shown). It is not found concentrated along stress fibers; indeed, on occasions less fluorescent areas are visible as dark lines running along the cell and these often appear coincident with actin microfilament bundles (an example is marked with an arrow, Fig. 3 *B* and *C*). The brain spectrin appears concentrated around the nucleus in many cells and is also enriched in ruffling membranes (Fig. 3 *G*). Notably, however, it does not appear enriched at the ends of the microfilament bundles in the adhesion plaque regions (see Fig. 3 *B* and *C*).

Cross-sections of tissues reveal this antigen at the periphery of cells (data not shown) as has been shown by Levine and Willard (23). An indication of this distribution at the cell's plasma membrane is found in Fig. 3 *J* and *L*, where primary cultures of adrenal medulla cells have been stained. These more spherical cells show an intense concentration of the antigen at or close to the plasma membrane (Fig. 3 *J*, *L*).

Brain Spectrin Causes the Gelation of F-actin Solutions

In initial studies using sedimentation analysis we could demonstrate that the brain protein bound F-actin. Since, however, a fraction of the brain spectrin tended to sediment in the absence of F-actin we chose to study the interaction with actin by low-shear, falling ball viscometry (25). The results are shown in Fig. 4. With increasing concentrations of the brain spectrin there was a marked increase in apparent viscosity of the actin solution (0.2 mg/ml) until complete gelation occurred at a concentration of ~60 μg/ml of the brain spectrin (an approximate molar ratio of 80:1, actin monomers: spectrin tetramers, or 40:1 if the spectrin is dimeric).

Brain Spectrin Binds to Erythrocyte Membranes

The interaction of the brain spectrin (fodrin) with inverted erythrocyte membrane vesicles was investigated using the binding assay of Bennett and Branton (3) modified as described above (see Materials and Methods). In a first set of experiments we investigated whether radioiodinated brain spectrin (2 μg/ml) could be detected binding to increasing concentrations of inverted vesicles (Fig. 5). Increased sedimentation of the labeled protein was observed with increasing concentrations of membranes and these values were significantly greater than in control experiments using the same protein but heated for 10 min at 55°C. These results were compared with the binding of ^{125}I-erythrocyte spectrin (1 μg/ml) in parallel experiments. With erythrocyte spectrin, a higher percentage of the total counts could be bound and relatively less binding was observed with the heat-treated samples. With increasing concentration

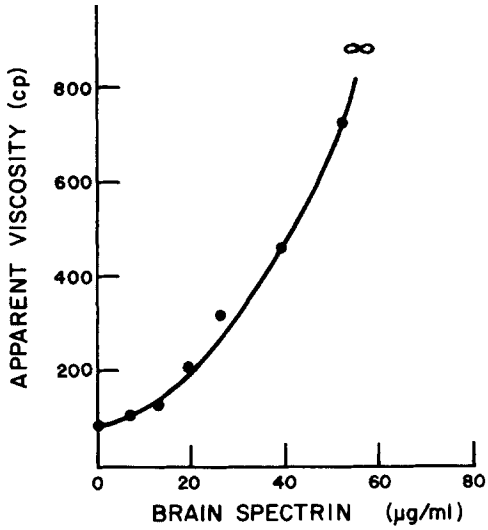

FIGURE 4 Effect of increasing brain spectrin concentration on the apparent viscosity of F-actin. Brain spectrin was added to F-actin (0.2 mg/ml). The final ionic conditions were 55 mM KCl, 1.6 mM $MgCl_2$, 0.02 mM EDTA, 0.02% β-mercaptoethanol, 10 mM Tris-Cl, pH 7.5. Apparent viscosity was measured at 21°C 1 h after mixing and drawing the samples into the capillary tube. F-actin alone had an apparent viscosity of 84 cp. Complete gelation of the actin-spectrin mixture occurred at concentrations of brain spectrin of 60 μg/ml and above.

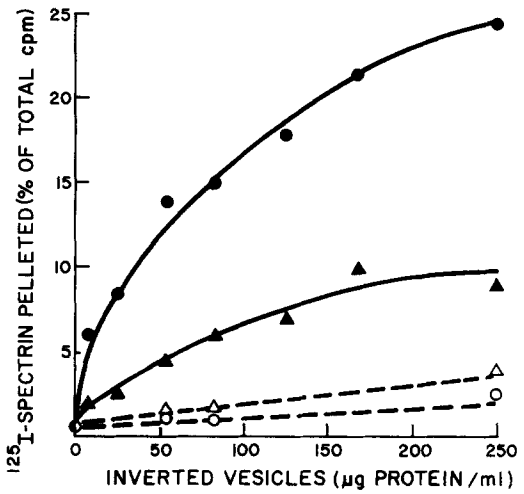

FIGURE 5 Binding of ^{125}I-erythrocyte and ^{125}I-brain spectrins to increasing concentrations of spectrin-depleted human erythrocyte inverted vesicles. ^{125}I-Erythrocyte spectrin (—●—: 1 μg/ml, 3.67 × 10^6 cpm/μg) or ^{125}I-brain spectrin (—▲—: 2 μg/ml; 1.35 × 10^6 cpm/μg) were incubated for 90 min at 4°C in 50 μl containing 2.5 mM sodium phosphate buffer, pH 7.6, 20 mM KCl, 1 mM $MgCl_2$, 0.1% β-mercaptoethanol, 1% BSA and various amounts of membrane protein. Some membrane vesicles were incubated with heat-denatured (10 min at 55°C) ^{125}I-erythrocyte spectrin (- - -○- - -), or ^{125}I-brain spectrin (- - -△- - -). Radioactivity was measured after centrifugation as described in Materials and Methods. Note that in the absence of any added membrane, <1% of the total counts were recovered in the bottom of the centrifuge tube.

of membranes both the erythrocyte and brain proteins appeared to plateau in the amount of protein that could bind to these membranes. The reason for this is not clear, but we suspect that this may be due to partial denaturation of the proteins during their preparations, iodination, or storage. Oth-

ers have reported that binding of erythrocyte spectrin to erythrocyte membranes decreases rapidly during storage (3).

The time course of the binding of brain and erythrocyte proteins to inverted vesicles is similar to that described previously for ^{32}P-erythrocyte spectrin binding (3). A steady-state was obtained after a 60-min incubation at 4°C (data not shown). Whether binding to the membranes was at the same site for both proteins was investigated in a series of competition experiments (Fig. 6). ^{125}I-brain spectrin and ^{125}I-erythrocyte spectrin were incubated with membranes together with increasing concentrations of unlabeled native proteins. As shown in Fig. 6 (upper panel) ^{125}I-brain spectrin binding is displaced competitively not only by increasing concentrations of unlabeled brain spectrin (apparent K_d of 200 μg/ml), but also by increasing concentrations of unlabeled erythrocyte spectrin (apparent K_d of 240 μg/ml). Conversely ^{125}I-erythrocyte spectrin binding (Fig. 6, lower panel) can be displaced by either erythrocyte spectrin (apparent K_d of 280 μg/ml) or by brain spectrin, surprisingly with a lower apparent K_d (220 μg/ml).

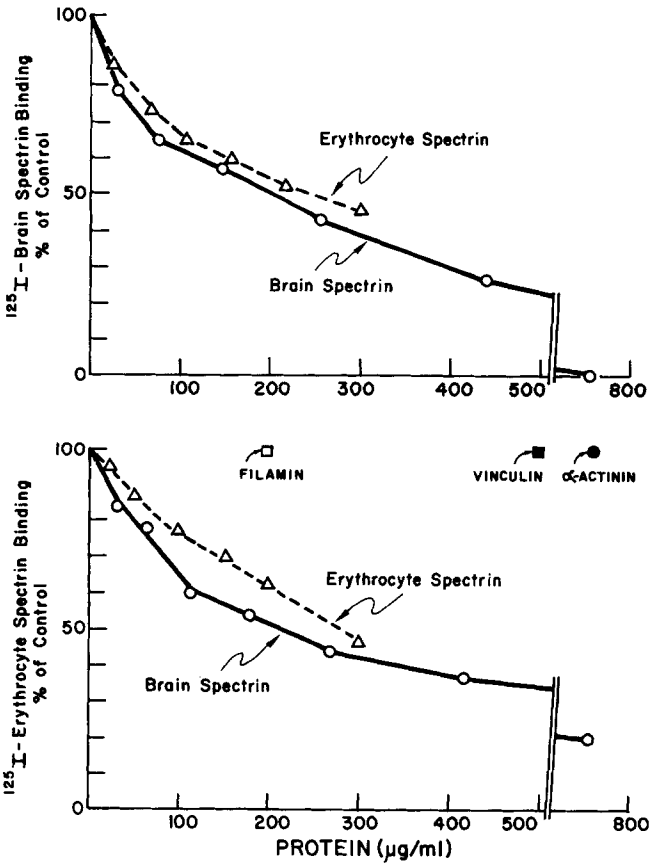

FIGURE 6 Competitive displacement of ^{125}I-brain spectrin and ^{125}I-erythrocyte spectrin binding to inverted vesicles with unlabeled brain and erythrocyte spectrins. ^{125}I-brain spectrin (2 μg/ml) (upper panel) or ^{125}I-erythrocyte spectrin (0.3 μg/ml) (lower panel) were incubated for 90 min at 4°C in 50 μl of binding buffer, containing 83 μg/ml of inverted vesicles and various concentrations of either unlabeled brain spectrin (○) or unlabeled erythrocyte spectrin (△). Incubation was started by addition of the inverted vesicles. Membrane-bound radioactivity was determined (see Materials and Methods) and the data were corrected for nonspecific binding, either by subtracting the values obtained from control assays using heat-denatured (10 min at 55°C) ^{125}I-erythrocyte spectrin (lower panel), or by subtracting the values obtained in the presence of 756 μg/ml of brain spectrin (upper panel). In the latter case, competition with this large excess of unlabeled brain spectrin resulted in a lower number of counts recovered in the pelleted membranes compared to controls using heat treated brain spectrin (suggesting that this heat treatment did not fully denature the brain spectrin). In both cases, 100% of binding corresponds to the value of binding obtained without any unlabeled purified protein added, that is, 2–3% of the total counts for ^{125}I-brain spectrin and 10–12% of total counts for ^{125}I-erythrocyte spectrin binding. Values plotted are the mean of four different experiments, each performed in duplicate using three different preparations of brain and erythrocyte spectrins.

The specificity of binding was tested using several other cytoskeletal proteins in competition experiments (Fig. 6, lower panel). Filamin at 200 μg/ml, vinculin at 500 μg/ml, and α-actinin at 700 μg/ml were unable to displace the binding of ^{125}I-erythrocyte spectrin to the membranes. Similarly, the binding was unaffected by the 1% BSA included in the assay or by the crude immunoglobulin fractions of preimmune antisera included in the assay in control experiments with antibodies against ankyrin (Table I).

Replotting the respective binding data of ^{125}I-brain spectrin with increasing concentration of brain spectrin, and of ^{125}I-erythrocyte spectrin with increasing concentration of erythrocyte spectrin, the binding of both proteins to the inverted membranes is saturable (plots not shown). In addition, Scatchard plot analysis (27) (Fig. 7) of the same data indicates that both proteins bind to a single class of binding site. From this analysis a dissociation constant value of 166 μg/ml has been determined for the brain spectrin with a maximal binding capacity of 80 μg brain spectrin per milligram of membrane protein. For erythrocyte spectrin a K_d value of 230 μg/ml was extrapolated with a maximal binding capacity of 540 μg/mg of membrane protein.

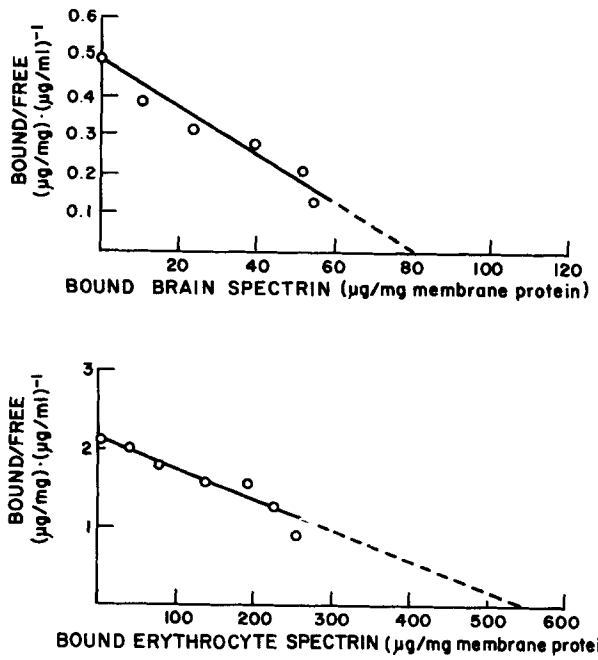

FIGURE 7 Scatchard plot analysis of the binding of ^{125}I-brain spectrin and ^{125}I-erythrocyte spectrin to inverted vesicles. The values obtained in Fig. 6 for ^{125}I-brain spectrin binding and corresponding displacement by the unlabeled brain spectrin, and for ^{125}I-erythrocyte spectrin binding and respective displacement by the unlabeled erythrocyte spectrin were treated according to the Scatchard equation (27), $B/F = nK - BK$, where B is micrograms of brain spectrin bound (upper plot) or erythrocyte spectrin (lower plot) per milligram of membrane protein, F is the respective concentration of unbound protein, n is the total number of binding sites (μg/mg) and K is the equilibrium constant (μg/ml)$^{-1}$.

TABLE I

Inhibition of Spectrin Binding to Inverted Erythrocyte Vesicles by Antibodies against Ankyrin

	^{125}I-Erythrocyte spectrin binding		^{125}I-Brain spectrin binding	
	cpm bound	*%*	*cpm bound*	*%*
Control	11,623	100	2,606	100
Preimmune 5F (2.9 mg/ml)	10,836	93	2,445	94
Immune 5Fa (6 mg/ml)	5,496	47	1,033	40
Preimmune 2F (10 mg/ml)	12,368	106	2,783	107
Immune 2Fa (16 mg/ml)	4,383	38	1,429	55
Immune 2Fb (10 mg/ml)	7,624	66	1,930	74

Effect of ankyrin antibodies on the binding of brain and erythrocyte spectrins to inverted vesicles. 10 μl inverted vesicles (0.42 mg/ml in binding buffer) were added to 30 μl of an immunoglobulin fraction of preimmune or immune sera from rabbits immunized with the 72,000-dalton proteolytic fragment of ankyrin. These immunoglobulin fractions were equilibrated in binding buffer by gel filtration immediately before use. Two different immunized rabbits (2F and 5F) were used, bleeds 2Fa and 5Fa were the first bleeds after immunization; 2Fb was a second bleed 5 wk later. After 30 min of preincubation at 4°C, 10 μl of ^{125}I-erythrocyte spectrin (0.3 μg/ml final volume) or ^{125}I-brain spectrin (2 μg/ml final volume) were added and the incubation was continued for an additional 90 min at 4°C. Membrane-bound radioactivity was determined as described in Materials and Methods. The values have been corrected for nonspecific binding determined in control assays, either with heat-treated ^{125}I-erythrocyte spectrin or by binding in the presence of 756 μg/ml of purified brain spectrin which resulted in lower values than with heat-treated brain spectrin. In control assays, preincubation was performed using 30 μl of binding buffer.

Anti-ankyrin Inhibits Binding of Brain Spectrin to Erythrocyte Membranes

To investigate in more detail the membrane binding site for brain spectrin we examined the effects of antibodies directed against ankyrin on the binding of both erythrocyte and brain spectrins to the inverted vesicles. Two different rabbit antisera were prepared against the 72,000-dalton proteolytic fragment of human erythrocyte ankyrin (1, 4). The characterization by immunoautoradiography of one of these antisera (rabbit 5F) is shown in Fig. 8. The antibody does not bind to spectrin (lanes *4* and *4'*), but labels strongly the 72,000-dalton fragment (lanes *3* and *3'*), as well as high molecular weight components in the inverted erythrocyte vesicle preparation (lanes *2* and *2'*) cor-

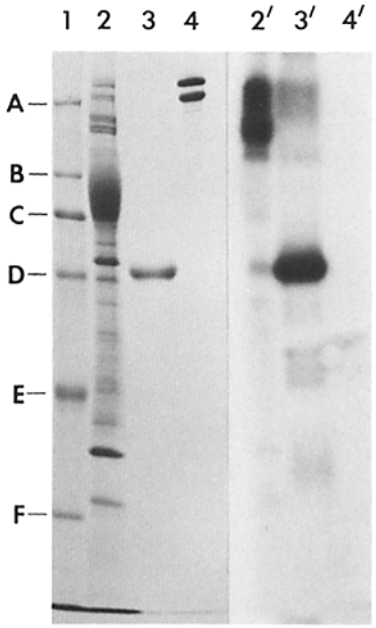

FIGURE 8 Immunoautoradiographic gel analysis of anti-ankyrin antiserum. Lanes *1–4* show a Coomassie Blue-stained SDS polyacrylamide gel on which were electrophoresed molecular weight marker proteins (lane *1*) (see Fig. 1 for details), a sample of erythrocyte inverted membrane vesicles used in the binding studies (lane *2*), the 72,000-dalton ankyrin fragment (lane *3*), and erythrocyte spectrin (lane *4*). Lanes *2'* to *4'* show the autoradiograph corresponding to a parallel gel slice (without the molecular weight markers) which was stained first with the antibody against the 72,000-dalton ankyrin fragment, followed by a second radioiodinated antibody directed against the first. Note the absence of labeling of the spectrin doublet (lane *4'*). In lane *3'*, the higher molecular weight bands (not detected by Coomassie Blue) probably represent aggregates of the ankyrin fragment. Several high molecular weight bands are labeled by the antibody in the inverted vesicle preparation (lane *2'*), which we take to correspond to intact ankyrin and proteolytic degradation products (29).

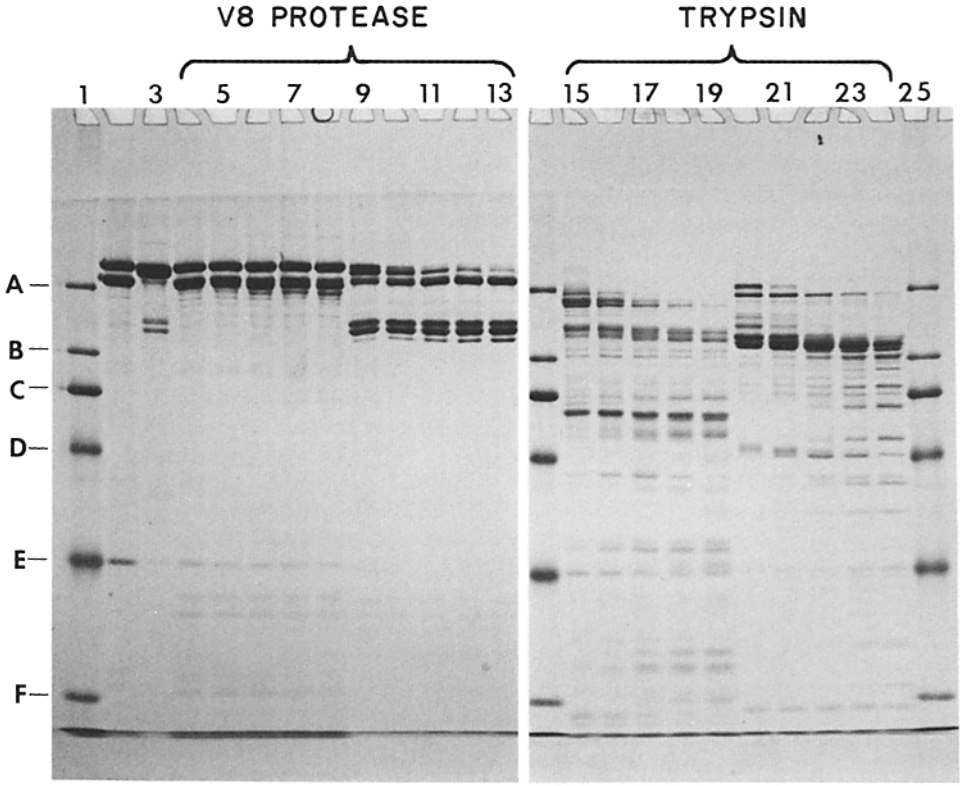

FIGURE 9 SDS gel analysis of partial proteolytic digestions of native erythrocyte and brain spectrins. Molecular weight marker proteins were electrophoresed in lanes *1*, *14*, and *25* (for details see Fig. 1). In lanes *2* and *3*, 6 μg of the undigested erythrocyte and brain spectrins were electrophoresed. Lanes *4–8* and *9–13* show 6 μg each of the staphylococcal V8 protease digestions of the erythrocyte and brain spectrins respectively, whereas lanes *15–19* and *20–24* show 6 μg each of the tryptic digestions of the erythrocyte and brain spectrins, respectively. Successive gel lanes for each enzyme represent successive time points at which the digestions were stopped (7, 14, 24, 35, and 50 min).

responding to native ankyrin (molecular weight 215,000 [4]) together with proteolytic breakdown products of lower molecular weight that have been observed by others (29).

Crude immunoglobulin fractions of these antisera and preimmune bleeds were tested for their effects on erythrocyte and brain spectrin binding after preincubation of the inverted vesicles for 30 min with the various antibody preparations (Table I). Both antisera significantly decreased the binding of ^{125}I-brain spectrin and ^{125}I-erythrocyte spectrin, while the preimmune fractions did not affect the binding. A second bleed of one of the rabbits (2F) taken 5 wk after the first bleed was also tested (2Fb). This lower titer antibody also decreased the binding of both proteins to the inverted vesicles, but to a lesser extent than the antibodies from the first bleeds (Table I).

Structural Comparison of Brain and Erythrocyte Spectrins by Native Proteolytic Digestion

Because of the many similarities between the brain and erythrocyte spectrins we have used partial proteolytic digestion to compare the structures of the native proteins. The SDS polyacrylamide gel patterns of the polypeptides generated by digestion of the two proteins with staphylococcal V8 protease and trypsin for increasing times are illustrated in Fig. 9. The patterns of digestion with the two enzymes are very different. Erythrocyte spectrin is largely unaffected by V8 protease at this concentration, but the brain protein is rapidly cleaved to a major band at 220,000 mol wt and a doublet at about 160,000 and 150,000 mol wt. These polypeptides appear resistant to further digestion during the course of the experiment. The relative resistance of both proteins to further digestion by V8 protease is consistent with the two proteins having similar structures. On the other hand, partial digestion of these proteins with trypsin (lanes *15–24*) or chymotrypsin (not shown) gives rise to rather different polypeptide patterns. Some similarities can be seen, however; in particular, a major group of polypeptides is generated with apparent molecular weights between about 160,000 and 140,000 for both proteins, possibly indicating similar domains in their native structures.

DISCUSSION

This paper concerns a high molecular weight actin-binding protein, the properties of which suggest it has a role in the attachment of actin to the plasma membrane of many cell types. This same protein was described recently by Levine and Willard (23) who named it fodrin and we show here that it has structural and functional similarities to erythrocyte spectrin. As such it probably corresponds also to the protein recently detected immunologically in nonerythroid cells with antibodies against spectrin (16). It probably also corresponds to the high molecular weight calmodulin-binding protein isolated from brain (12). The structural and functional properties of the brain protein are sufficiently similar to those of erythrocyte spectrin that we suggest they belong to the same family of proteins and that it may be appropriate to refer to them all by the same name (spectrin) prefaced by the tissue type rather than coining a new name for each one that is isolated. The myosin family of polypeptides serves as an example where initially new names were coined for the equivalent nonmuscle proteins, but where subsequent work demonstrated sufficient structural and functional homologies to justify calling them all myosin, even though some do not show immunological cross-reaction. Similarly with this class of proteins it would seem probable that they are related structurally and functionally, having evolved from a common precursor, with erythrocyte spectrin possibly being the most specialized form.

Brain spectrin has many properties in common with erythrocyte spectrin. Some are also shared with the class of high molecular weight actin-binding proteins that includes filamin and macrophage ABP (18, 19, 28). For example, they are all high molecular weight, asymmetric proteins in solution, and they all cross-link actin filaments. Other properties of the spectrins, however, distinguish them from this filamin/ABP class of proteins. On SDS polyacrylamide gels the spectrins reveal two distinct high molecular weight polypeptides compared with the single band of filamin or ABP. Brain and erythrocyte spectrins show a characteristic shift in S value in the presence of 20 mM KCl (Fig. 2). Standard proteins show little or no shift, whereas filamin also shows a slight shift in S value but this is less than for the spectrins. The significance of this shift is not clear. The brain and erythrocyte spectrins cross-react immunologically. This cross-reaction is weak and was not initially detected by us until we exposed autoradiographs such that the gel track corresponding to brain spectrin was overexposed (Fig. 1). Of particular significance, both spectrins show mutually competitive binding to inverted erythrocyte membranes (Fig. 6), whereas no competitive binding was observed with filamin, α-actinin, or vinculin. The binding of both the brain and erythrocyte proteins to erythrocyte membranes is inhibited by antibodies against ankyrin (Table I), indicating that both are associating with the erythrocyte membrane via ankyrin. Based on these similarities we would suggest that the brain protein and erythrocyte spectrin are related structurally and functionally, though clearly they are not identical, having slightly different subunit molecular weights and differing significantly in their native partial peptide maps (Fig. 9).

Since completing this work, Glenney et al. (14) have presented evidence on another similar protein which they isolated from the terminal web of the brush border and which they refer to as TW 260/240. They have compared this protein with brain spectrin (fodrin) and erythrocyte spectrin and have shown similarities between these proteins, particularly between TW 260/240 and brain spectrin. Initially immunological cross-reaction between these two proteins was demonstrated, but cross-reaction with erythrocyte spectrin was not. In a second paper (15), that has appeared after this present study was submitted for publication, they have demonstrated immunological cross-reaction with erythrocyte spectrin, as we have shown here for the brain and erythrocyte spectrins. All three proteins were shown by Glenney et al. (14) to have a very similar appearance when single molecules were examined by electron microscopy and their appearance was distinct from that of filamin. Similar results with brain spectrin (fodrin) and erythrocyte spectrin have also been obtained by Bennett and co-workers, who have also found immunological cross-reaction between these two proteins (Bennett, personal communication and manuscript in press). Glenney et al. (14) also found that TW 260/240, brain and erythrocyte spectrin bound calmodulin in a calcium dependent fashion, but filamin did not. This binding of calmodulin suggests that the brain protein described by Davies and Klee (12) is almost certainly brain spectrin, being also a doublet on gels and binding actin. However, these authors were unable to demonstrate immunological cross-reaction with antibodies to human erythrocyte spectrin, which probably reflects the narrow specificity of most antisera made against human erythrocyte spectrin. Similarly, Glenney et al. initially classified erythrocyte spectrin in a distinct group from TW 260/240 and brain fodrin (14), but their more recent

demonstration of immunological cross-reactivity (15) has led them to group these proteins together, as we have done here. In this most recent paper (15) they have provided evidence for the similarity of the 240,000-dalton subunits of brain fodrin, erythrocyte spectrin, and the corresponding brush border protein subunit based on partial peptide maps of the SDS-denatured polypeptides. The similarity of these peptide maps is quite pronounced and somewhat surprising given the rather different peptide maps that we have found with the native proteins (Fig. 9).

Nonerythroid spectrins occur in many cell types. Using antibodies against the brain protein and immunofluorescence or immuno-autoradiography on gels, we have identified spectrin in mouse, gerbil, and chicken fibroblasts, in Hela cell plasma membranes and in the chromaffin cells of the adrenal medulla (Figs. 1 and 3), as well as in monocytes, cultured kidney epithelial cells and smooth muscle (data not shown). Levine and Willard (23) have demonstrated the same antigen in several whole tissues (kidney, liver, vas deferens, heart, skeletal muscle, and intestine) as well as in cultured neurons and fibroblasts. Thus it seems that this family of proteins is found in most, possibly all, higher vertebrate cell types.

The similarity of the nonerythroid spectrin to spectrin from erythrocytes argues for a parallel function of actin attachment to membranes in other cells as well. The localization of spectrin close to the plasma membrane of many cell types (23) and the demonstration here that brain spectrin will bind to erythrocyte membranes and compete in this binding with erythrocyte spectrin supports this idea strongly. It will be important in future work to determine how much of the actin in nonerythroid cells is linked to the membrane via spectrin and how much via other proteins. In this regard, it should be noted that although it is localized over the surface of cells with a mottled distribution, it is not concentrated in adhesion plaques, regions where bundles of microfilaments terminate and associate with the plasma membrane. Spectrin could function in such locations, but it seems likely that the nature of the actin attachment in these regions will be different. In adhesion plaques attachment may be via the ends of actin filaments whereas elsewhere much of the attachment to the plasma membrane may be through lateral associations along the lengths of actin filaments. In future work it will also be important to determine whether spectrin is linked to these nonerythroid cell membranes via ankyrin as it is in the erythrocyte (4). Our finding that the binding of brain spectrin to erythrocyte membranes is inhibited by antibodies against ankyrin indicates that at least an ankyrin-binding site on the protein has been conserved. Also ankyrin has been detected immunologically in other cell types (2). may be applicable to other cells as well.

The authors are grateful to Laurie Connell for help with many aspects of the work, Teresa Dinse for preparation of the manuscript, and Marion Blackburn for help with preparation of the figures. The authors thank the monitoring editor for many helpful suggestions.

P. Mangeat was supported by a NATO fellowship. The work was supported by National Institutes of Health grant GM 29860 and a grant from the Muscular Dystrophy Association.

Received for publication 24 May 1982, and in revised form 10 August 1982.

Noted Added in Proof: Using a calmodulin affinity column we have recently confirmed that brain spectrin is the high molecular weight polypeptide doublet previously described by Davies and Klee (12). The widespread occurrence of nonerythrocyte spectrins in avian and mammalian cells has also been described recently by two other groups showing immunological cross-reaction with antibodies against either chicken erythrocyte α spectrin (Repasky, E. A., B. L. Granger, and E. Lazarides, 1982, *Cell* 29:821–833) or human erythrocyte spectrin (Bennett, V., J. Davis, and W. E. Fowler, 1982, *Nature (Lond.)*. 299:126–131). This latter group also demonstrated the ability of brain spectrin to bind to erythrocyte membranes as we have shown here.

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