Brain Ankyrin

PURIFICATION OF A 72,000 M₀ SPECTRIN-BINDING DOMAIN

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Polypeptides of M₀ = 190,000–220,000 that cross-react with erythrocyte ankyrin were detected in immunoblots of membranes from pig lens, pig brain, and rat liver. The cross-reacting polypeptides from brain were cleaved by chymotrypsin to fragments of M₀ = 95,000 and 72,000 which are the same size as fragments obtained with erythrocyte ankyrin. The brain 72,000 M₀ fragment associated with erythrocyte spectrin, and the binding occurred at the same site as that of erythrocyte ankyrin 72,000 M₀ fragment since (a) brain 72,000 M₀ fragment was adsorbed to erythrocyte spectrin-agarose and (b) ¹²⁵I-labeled erythrocyte spectrin bound to brain 72,000 M₀ fragment following transfer of the fragment from a sodium dodecyl sulfate gel to nitrocellulose paper, and this binding was displaced by erythrocyte ankyrin 72,000 M₀ fragment. Brain 72,000 M₀ fragment was purified about 400-fold by selective extraction and by continuous chromatography on columns attached in series containing DEAE-cellulose followed by erythrocyte spectrin coupled to agarose, and finally hydroxylapatite. The brain 72,000 M₀ fragment was not derived from contaminating erythrocytes since peptide maps of pig brain and pig erythrocyte 72,000 M₀ fragments were distinct. The amount of brain 72,000 M₀ fragment was estimated as 0.28% of membrane protein or 39 pmol/mg based on radioimmunooassay with ¹²⁵I-labeled brain fragment and antibody to erythrocyte spectrin. Brain spectrin tetramer was present in about the same number of copies (30 pmol/mg of membrane protein) based on densitometry of Coomassie blue-stained sodium dodecyl sulfate gels. The binding site on brain spectrin for both brain and erythrocyte ankyrin 72,000 M₀ fragments was localized by electron microscopy to the midregion of spectrin tetramers about 90 nm from the near end and 110 nm from the far end. These studies demonstrate the presence in brain membranes of a protein closely related to erythrocyte ankyrin, and are consistent with a function of the brain ankyrin as a membrane attachment site for brain spectrin.

The human erythrocyte membrane is currently the best understood system in terms of knowledge of the organization of its membrane and cytoskeletal proteins (see reviews by Branton et al., 1981; Bennett, 1982). The major integral membrane protein, which contains an anion channel, is associated on the cytoplasmic surface of the membrane with ankyrin. Ankyrin, in turn, is attached through a 72,000 M₀ domain to spectrin which is a flexible rod-shaped protein 180 nm in length composed of two subunits. The subunits of spectrin are aligned side-to-side to form heterodimers, and the dimers are assembled by head-to-head association to form tetramers. Spectrin tetramers bind to their ends to a protein named band 4.1 and to actin oligomers. Spectrin tetramers with associated actin and band 4.1 form a two-dimensional network that lines the inner surface of the plasma membrane and provides mechanical stability for the fragile lipid bilayer.

Analogns of erythrocyte membrane proteins are widely distributed in other cell types. Nonerythroid spectrin was initially identified on the basis of cross-reaction with antibody against erythrocyte spectrin (Goodman et al., 1981; Repasky et al., 1982; Bennett et al., 1982a; Burridge et al., 1982). Immunoreactive forms of band 4.1 have also been detected in other cells (Cohen et al., 1982). Brain spectrin has been purified and demonstrated to have properties quite similar to erythrocyte spectrin, including two subunits arranged as a tetramer with the morphology of a flexible rod 200 nm in length, and binding sites for actin, band 4.1 and ankyrin (Bennett et al., 1982a, Burridge et al., 1982; Glenney et al., 1982; Burns et al., 1983; Lin et al., 1983). Subunits of brain spectrin tetramers are arranged the same as those of erythrocyte spectrin, and it is possible to prepare functional hybrids of subunits of brain and erythrocyte spectrin (Davis and Bennett, 1983).

Immunoreactive forms of erythrocyte ankyrin have been detected by radioimmunooassay in membranes and whole cells from a variety of tissues (Bennett, 1979). The polypeptides cross-reacting with erythrocyte ankyrin include microtubule-associated proteins localized in the cytoplasm and in mitotic structures (Bennett and Davis, 1981; Bennett et al., 1982b). In addition, nonerythroid cells contain membrane-associated polypeptides of M₀ ~ 200,000 that cross-react with ankyrin (Bennett et al., 1982b). This report describes further studies with membrane-associated forms of ankyrin from brain, including purification of a 72,000 M₀ fragment that binds to spectrin. Brain ankyrin is present in approximately the same quantities as brain spectrin tetramers and is a logical candidate for a membrane-attachment site for brain spectrin. These studies provide additional support for the view that the organization of the erythrocyte membrane will have direct relevance for other cells.

EXPERIMENTAL PROCEDURES

Materials—Na¹³¹I was from Amersham. Hydroxylapatite (fast flow), Protein A-bearing staphylococci, and biotin-N-hydroxysucc-
cinobide ester were from Calbiochem-Behring. Plastic thin layer sheets coated with 0.1-mm thick cellulose were from E. Merck. PMSF, DFP, DTT, pepstatin A, leupeptin, Triton X-100, and pancreatic trypsin inhibitor were from Sigma. Nitrocellulose paper and electrophoresis reagents were from Bio-Rad. Sucrose, urea, and ammonium sulfate were from Schwarz/Mann. α-Chymotrypsin (54 units/mg) was from Worthington. Cyanogen bromide-activated Sepharose 4B, Protein A, Protein A-Sepharose, and Sephacryl S-500 were from Pharmacia. Avidin-ferritin was from LKB. Pig erythrocyte spectrin was purified by chromatography on a Sephacryl S-500 column as described (Bennett, 1983). Ankyrin 72,000 M, fragment was purified from pig and human erythrocytes as described (Bennett, 1977). Affinity-purified rabbit antibody against erythrocyte ankyrin was prepared as described (Bennett and Davis, 1982). Preimmune Ig was isolated by affinity chromatography on Protein A-Sepharose, using the same elution conditions as for immune antibody. Pig brains were obtained from a local slaughter house; tissue from the cerebral cortex was dissected free of connective tissue, washed with 0.25 M sucrose and frozen in liquid nitrogen. Frozen brain was stored at −100 °C and used within 6 weeks.

Methods—Tissues except for liver were homogenized with a Brinkman Polytron (large head) for 30–60 s at a setting of 5.5. Liver was disrupted in a Dounce homogenizer. SDS-polyacrylamide electrophoresis was performed on 3.5–17% exponential gradient slab gels with the buffers of Fairbanks et al. (1971). Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard. The E"_2₈₀ of brain ankyrin 72,000 M, fragment was 9.0 based on protein determination by Bradford assay, and A₂₈₀ was used to estimate protein with purified preparations as a standard. Autoradiography was performed with X-Omat AR film (Kodak) and Cronex intensifier screens (DuPont). Proteins were radiiodinated with Na-¹²⁵I using chloramine-T as an oxidant (Hunter and Greenwood, 1962).

Immunoblot analysis was performed by electrophoretically transferring proteins from SDS gels to nitrocellulose paper (Towbin et al., 1979) using conditions as described (Davis and Bennett, 1982). The nitrocellulose paper was incubated 15 min at 24 °C in immunoblot buffer (40 mg/ml of bovine serum albumin, 150 mM NaCl, 10 mM sodium phosphate, 1 mM Na EDTA, 1 mM NaN₃, 0.2% (v/v) Triton X-100, pH 7.5) and then for 1 h at 4 °C with the same buffer and 0.5–1 μg/ml of antibody. The nitrocellulose paper was washed 5 times with buffer (no albumin) and once with 2 ml urea, 0.1 M glycine, 1% Triton X-100. The bound antibody was incubated for 2 h at 4 °C with ¹²⁵I-labeled Protein A (0.5–1.5 CI/μmol; 10⁶ cpm/ml final concentration) in immunoblot buffer. The nitrocellulose was washed as before and dried and an autoradiogram was prepared.

Pig erythrocyte spectrin-agrose was prepared by addition of cyanogen bromide-activated Sepharose 4B to an equal volume of solution containing 2 mg/ml of spectrin, 25 mM sodium phosphate, 750 mM NaCl, pH 8. The suspension was mixed gently at 4 °C for 2 h, and then poured into a column and washed with 1 liter of 1 M NaCl, 0.1 M glycine, 0.5% Triton X-100, 1 mM NaN₃. The gel was then washed with 10 volumes of 0.1 M NaCl, 10 mM sodium phosphate, 1 mM NaN₃, and stored in this buffer. The affinity column was regenerated after use by washing with the high salt/Triton X-100 buffer and could be used twice.

RESULTS

Identification of Membrane-associated Polypeptides Cross-reacting with Erythrocyte Ankyrin—A polypeptide of Mr = 190,000 cross-reacting with erythrocyte ankyrin has been identified in rat liver plasma membranes by the immunoblot technique (Bennett et al., 1982b). Membranes from pig brain, pig lens, and rat liver were analyzed by the same method (Fig. 1). Lens membranes contain a major cross-reacting polypeptide of Mr = 220,000 and many polypeptides of lower Mr. Brain membranes contain two major cross-reacting polypeptides of Mr = 220,000 and 215,000, and a minor polypeptide of Mr = 190,000. The relative amounts of the Mr = 220,000, 215,000, and 190,000 polypeptides varied in different preparations, depending on the presence of protease inhibitors and time required to isolate membranes. The Mr = 215,000 and 190,000 polypeptides may be proteolytic products of the Mr = 220,000 or may represent related isoforms with some common sequence but which are products of different genes. Liver plasma membranes contained a major polypeptide of Mr = 190,000 and a fainter cross-reacting band at Mr = 215,000. Control immunoblots with preimmune Ig labeled no detectable polypeptides (Fig. 1). Such controls with preimmune Ig were negative in other immunoblot experiments (Figs. 2–5) and are not shown.

The polypeptides cross-reacting with erythrocyte ankyrin are especially sensitive to exogenous protease (see below) as well as tissue proteases. The gels presented here were the best of several experiments, and were obtained with membranes isolated rapidly and with protease inhibitors DFP, leupeptin, pepstatin A, PMFSF, and EGTA (to inhibit Ca²⁺-dependent protease). The cross-reacting polypeptides were especially sensitive to a leupeptin-inhibited protease activity. Lens membranes exhibited multiple cross-reacting bands in spite of these precautions, and it is possible some degradation occurred in vivo.

Brain membranes were chosen for further studies since these can be obtained in large quantities. A crude subcellular fractionation of brain indicated that the cross-reacting Mr = 220,000 and 215,000 polypeptides were confined almost entirely to particulate fractions (Fig. 2). The cross-reacting polypeptides co-migrated with the major peak of membrane
protein when membranes were fractionated by isopycnic centrifugation on sucrose gradients, but were deficient in the myelin fractions (Fig. 2). The distribution of these bands paralleled approximately that of the brain spectrin doublet \((M_r = 260,000 \text{ and } 265,000)\). It was difficult to compare exactly the amounts of spectrin and cross-reacting polypeptides because of the nonquantitative nature of immunoblots.

The cross-reacting polypeptides are associated tightly with membranes, since they were not extracted by repeated washes of 0.5 \(M\) NaCl which removed about 50\% of the spectrin (Fig. 3). Similarly, the polypeptides were not solubilized by extraction of membrane at low ionic strength which also entrapped spectrin (not shown).

Proteolysis of Brain Cross-reacting Polypeptides to a Membrane-associated Fragment of \(M_r = 95,000\) and a Spectrin-binding Fragment of \(M_r = 72,000\)—Erythrocyte ankyrin contains two major protease-resistant domains, one of \(M_r = 72,000\) which contains the spectrin-binding site (Bennett, 1978) and another of \(M_r = 95,000\) (Bennett and Stenbuck, 1980). The 95,000 \(M_r\) domain contains the anion channel binding site and remains associated with membranes after cleavage.2 An important criterion for nonerythroid ankyrin is that these proteins should have a similar domain structure. The brain cross-reacting polypeptides fulfill this requirement, since limited digestion with \(\alpha\)-chymotrypsin degraded the \(M_r = 220,000, 215,000, \text{ and } 190,000\) polypeptides to fragments of \(M_r = 95,000\) and 72,000 (Fig. 3). The 72,000 \(M_r\) fragment was extracted from the digested membranes with 0.5 \(M\) NaCl, while the major portion of the 95,000 \(M_r\) fragment remained membrane-bound (Fig. 3). The persistent binding of the 95,000 \(M_r\) fragment while the 72,000 \(M_r\) fragment was extracted suggests that the 95,000 \(M_r\) fragment is primarily responsible for attachment of the intact polypeptide to the membrane.

Experiments in Fig. 4 demonstrate that the solubilized brain 72,000 \(M_r\) fragment binds to erythrocyte spectrin at the same site as erythrocyte ankyrin 72,000 \(M_r\), fragment. The brain fragment was adsorbed to an erythrocyte spectrin affinity column and eluted onto a second column of hydroxyapatite (see below) resulting in substantial purification (Fig. 4). The association of the fragment with the affinity column most likely involved a direct association between the fragment and spectrin since the fragment transferred from SDS gels to nitrocellulose bound \(^{125}\)I-labeled erythrocyte spectrin under immunoblot conditions (Fig. 4). Several types of controls

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2 V. Bennett, unpublished data.
extracted prior to digestion with proteinase K, which permitted removal of protease and protein solubilized during proteolysis. The fragment then was extracted from digested membranes with 0.5 M NaCl, thus selecting for polypeptides released in high salt only after proteolysis. The extract of digested membranes was applied to an erythrocyte spectrin affinity column, which adsorbed the fragment. The fragment was recovered from the hydroxylapatite column by eluting with 0.4 M NaCl, which removed about 20% of the protein and little ankyrin (Fig. 3). The 72,000 M, fragment remained associated with membrane after the digestion with chymotrypsin, which permitted removal of protease and protein solubilized during proteolysis. The fragment then was extracted from digested membranes with 0.5 M NaCl, thus selecting for polypeptides released in high salt only after proteolysis. The extract of digested membranes contained about 80–90% of the fragment and only 4% of the protein (Table I).

The solubilized fragment was then purified by a one step procedure involving three different columns connected in series. The advantages of continuous chromatography are that loss of sample is minimized and the time for the procedure is reduced. The digest was applied first to a column of DE53—cellulose under conditions where the fragment was not adsorbed. The effluent from the DE53 column ran directly onto an erythrocyte spectrin affinity column which adsorbed the fragment. The fragment was gradually eluted from the affinity column with a large volume of loading buffer and collected directly on a small column of hydroxylapatite. The hydroxylapatite column was then eluted with a gradient of phosphate, and the fragment was obtained in the peak fractions.
Table I

<table>
<thead>
<tr>
<th>Fraction**</th>
<th>Protein (mg)</th>
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<tr>
<td>Washed membranes</td>
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<tr>
<td>0.5 M NaCl pre-extract</td>
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<td>Post chymotrypsin membranes</td>
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<tr>
<td>0.5 M NaCl extract</td>
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<tr>
<td>DE53 0.5 M NaCl cut</td>
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<tr>
<td>Spectrin-Sepharose breakthrough</td>
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<tr>
<td>Purified brain fragment</td>
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</tbody>
</table>

*For explanation, see Fig. 5.

Table II

<table>
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<th>Location of ferritin</th>
<th>Biotin-labeled brain fragment (1078 molecules counted)</th>
<th>Biotin-labeled red blood cell fragment (546 molecules counted)</th>
<th>Control (981 molecules counted)</th>
<th>% bound</th>
<th>% bound minus controls</th>
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</table>

For explanation, see Fig. 5.
apatite (Fig. 5). Other spectrin-binding proteins were eluted from the spectrin affinity column with 0.5 M NaBr, and these proteins also could be concentrated on a second column of hydroxylapatite (Fig. 5). The purified fragment was about 80% pure based on densitometry of Coomassie blue-stained SDS gels, and the yield from 250 g of brain tissue was 300 µg (Table II). The fragment was purified about 400-fold with a yield of only about 2% based on estimates of 2.8 µg of fragment/mg of membrane protein (see below). Major losses occurred due to the tendency of the fragment to adsorb to surfaces, especially in early stages of purification.

A major concern was that the 72,000 M₉ fragment might be derived from contaminating erythrocytes. This possibility was excluded by the fact that peptide maps of the brain and pig erythrocyte fragments are distinct with very few common peptides (Fig. 6). The peptide maps share some similarities in that the number of peptides are nearly the same, and that the pattern is similar. However, if peptides of each fragment are co-electrophoresed, it is clear that these are not identical peptides.

An initial problem in these studies was that some proteins in the crude digest, including fragment, formed a precipitate when dialyzed against low ionic strength (less than 10 mM NaCl). The precipitate was enriched in a polypeptide of M₉ = 130,000 cross-reacting with brain spectrin, 72,000 M₉ ankyrin fragment, and a polypeptide at 43,000 M₉, that may be actin. The requirement for salt during dialysis prevented conventional application of DEAE-chromatography since the fragment eluted from DE53-cellulose at 50-70 mM NaCl.

Estimate of the Amount of 72,000 M₉ Fragment in Brain Membranes—The pure ¹²⁵I-labeled fragment was used as a ligand in a radioimmunooassay to estimate the amount of 72,000 M₉ fragment and presumably ankyrin in brain tissue (Fig. 7). Binding of ¹²⁵I-labeled brain fragment was measured to antibody against erythrocyte ankyrin, and this binding was displaced in a parallel fashion by membrane protein solubilized in SDS to ensure accessibility to the antibody and by SDS-denatured fragment. The effects of SDS in the assay were minimal, and SDS at equivalent concentrations was present in control samples. Comparison of displacement of binding by fragment and membranes indicates the presence of fragment at about 0.28% of the total protein, or 39 pmol/mg of membrane protein. Brain spectrin tetramer was estimated in the same membranes to be present at 30 pmol/mg based on densitometry of Coomassie blue-stained gels.

A significant difficulty in performing these measurements was that anti-erythrocyte ankyrin Ig bound brain fragment with a relatively low affinity of 16 nM (not shown). Thus, the assay was relatively insensitive and required substantial amounts of brain membrane protein. High concentrations of membrane protein caused nonspecific interference with the assay, and for this reason the displacement curve in Fig. 7 was not extended above 70 µg/ml of membrane protein. More accurate measurements will require high affinity antibody raised against brain fragment. It is likely that the estimate of brain fragment from Fig. 7 is approximately correct since a similar value of 0.2% of the membrane protein as 72,000 M₉ fragment was obtained previously in a different assay (Bennett, 1979). The measurements in this earlier study were made by comparison of displacement of binding of ¹²⁵I-labeled erythrocyte 72,000 M₉ fragment to antibody against erythrocyte 72,000 M₉ fragment by rat erythrocytes and rat brain membranes.

Localization of the Binding Site on Brain Spectrin for Brain and Erythrocyte 72,000 M₉, Fragments—Binding of erythrocyte and brain ankyrin fragments to brain spectrin was visualized by rotary shadowing of spectrin molecules incubated with biotin-labeled fragments and then avidin-ferritin (Fig. 8). Biotin was coupled to the fragments using biotin-N-hydroxysuccinimide ester, and the reaction was monitored by the amount of fragment sedimented with avidin-ferritin. By this criterion, at least 90% of both fragments were conjugated to biotin.

Electron micrographs of rotary-shadowed replicas of brain spectrin tetramer incubated with biotin-labeled brain ankyrin fragment and avidin-ferritin in a 1:1 molar ratio demonstrated ferritin-labeling of 16% of spectrin molecules at a site in the midregion. This site was 90 ± 4 nM from the near end and 110 ± 5 nM from the far end (Fig. 8). Samples with erythrocyte ankyrin fragment exhibited labeling of 13% of spectrin molecules in the same midregion site. In addition to labeling at the midregion of spectrin, ferritin was ob-

FIG. 6. Two-dimensional maps of ¹²⁵I-labeled chymotrypsin peptides of pig erythrocyte ankyrin 72,000 M₉, fragment (A), brain ankyrin 72,000 M₉, fragment (B), and a mixture of peptides from both erythrocyte and brain fragments (C). Pig erythrocyte 72,000 M₉, fragment and brain 72,000 M₉, fragment (5 µg) were denatured in 0.05% (w/v) SDS, radiolabeled with 1 mCi of ¹²⁵I using chloramine-T as an oxidant, and electrophoresed on a SDS-polyacrylamide gel. The ¹²⁵I-labeled fragments were localized by staining with Coomassie blue, cut from the gel, and incubated 3 h at 37 °C in 50 mM ammonium acetate, 1 mM NaN₃, 50 µg/ml α-chymotrypsin followed by a 15-h incubation with an additional 50 µg/ml of enzyme. The digest was lyophilized and analyzed by electrophoresis (horizontal dimension) and chromatography (vertical dimension) as described (Elder et al., 1977; Davis and Bennett, 1982).
were added to tubes containing, in a final volume of 0.2 ml, 0.9 pg/ml of bovine serum albumin, 150 mM NaCl, 10 mM sodium phosphate, 1 pmol). The incubation was continued for
by displacement of binding
plexes were pelleted
ends and 3-fold more labeling than all other sites on the
midregion site exhibited 10-fold more labeling than at the

membranes. The criteria that seem appropriate at this point
served in 6% of molecules incubated with brain fragment at
the ends of spectrin tetramers, and in 6% of the molecules at
other sites (Table II). Control samples with avidin-ferritin
but no biotin-fragments exhibited labeling of 6% of spectrin
molecules approximately in the midregion, 5% of molecules
at the ends, and 5% at other regions along spectrin molecules.
Ferritin-labeling of the midregion of spectrin incubated
with biotin-labeled brain fragment, was specific by several
criteria. The site was labeled at a 3-fold lower frequency when
fragments were omitted. When labeling at the midregion,
ends, and other regions of the spectrin molecules was cor-
corrected for nonspecific labeling in the absence of fragments,
the midregion site exhibited 10-fold more labeling than at the
ends and 3-fold more labeling than all other sites on the
molecule combined (Table II).

DISCUSSION

This report describes identification of membrane-associated
polyptides of $M_r = 190,000$--$220,000$ in brain, lens, and
liver that cross-react with erythrocyte ankyrin. The cross-
reacting polyptides in brain, and most likely other tissues as
well, are closely related to erythrocyte ankyrin in structure and
function. The brain polyptides are digested by mild
proteolysis to domains of $M_r = 95,000$ and 72,000 (Fig. 3)
which are the same size as fragments obtained by digestion
of erythrocyte ankyrin (Bennett, 1978; Bennett and Stenbuck,
1980). The brain 72,000 M, fragment binds to erythrocyte
spectrin at the same site as erythrocyte 72,000 M, fragment
(Fig. 4) and was purified by affinity chromatography on
erythrocyte spectrin-agarose (Fig. 5). Purified brain 72,000
M, fragment was distinct from erythrocyte 72,000 M, frag-
ment by peptide maps (Fig. 6), but both fragments bound to
brain spectrin tetramer at a site in the midregion about 90
nM from the nearest end (Fig. 8). The ankyrin binding site

on erythrocyte spectrin has been localized to a similar region
on erythrocyte spectrin (Tyler et al., 1979). The amount of
brain 72,000 M, fragment was estimated by radioimmunoas-
say to be 0.28% of the membrane protein or 39 pmol/mg,
which is about the same as the number of brain spectrin
tetramers (30 pmol/mg of membrane protein).

These experiments provide strong evidence for the wide-
spread presence of proteins closely related to ankyrin in cell
membranes. The criteria that seem appropriate at this point

FIG. 7. Estimate of amount of ankyrin in brain membranes
by displacement of binding of $^{125}$I-labeled brain 72,000 M,
fragment to anti-erythrocyte ankyrin Ig. Pig brain membranes
(10 mg/ml) (Fig. 1) and purified brain 72,000 M, fragment (100 μg/
ml) were denatured in 1% (w/v) SDS, 5 mM Na EDTA, 20 mM DTT,
10 mM Tris, 50 μg/ml of PMSF, pH 7.5. Portions of these samples
were added to tubes containing, in a final volume of 0.2 ml, 0.9 μg/
ml of bovine serum albumin, 150 mM NaCl, 10 mM sodium phosphate, 1
mM Na EDTA, 1 mM NaN₃, 2 μg/ml of pancreatic trypsin inhibitor,
1% (v/v) Triton X-100, 0.2 μl of Protein A-bearing staphylococci,
and $^{125}$I-labeled brain ankyrin fragment (0.47 pmol, 1.4 $\times 10^6$ cpm/
ml). The incubation was continued for 3 h at 4 °C and then the
samples were diluted with 3 ml of 0.5 M NaBr, 1 mM glycine, 1% Triton
X-100. The staphylococci with adsorbed immune complexes were pelleted
(10 min, 2000 × g) and assayed for $^{125}$I. The data are presented as per cent binding in the presence of equivalent
amounts of SDS but without other additions, and are corrected for
nonspecific binding by subtracting the value obtained with nonimmun
Ig at each concentration of protein tested.

FIG. 8. Localization by electron microscopy of binding sites
on brain spectrin for brain and erythrocyte ankyrin 72,000
M, fragments. Biotinyl derivatives of brain and human erythrocyte
ankyrin 72,000 M, fragments were prepared by reaction for 1 h at
4 °C of 5 μM biotin N-hydroxysuccinimide ester with 1 μM fragment
in 25 mM sodium phosphate buffer, pH 8, followed by dialysis against
50 mM NaCl, 10 mM sodium phosphate, 1 mM NaBr, 0.5 mM DTT.
Brain spectrin and biotin-labeled fragments were incubated at 5 $\times
10^{-5}$ M of each protein for 14 h at 4 °C in 90 mM NaCl, 10 mM sodium
phosphate, 1 mM NaBr, 0.5 mM DTT, followed by incubation for 1 h
at 4 °C with avidin-ferritin at a final concentration of 5 $\times 10^{-5}$ M.
Control samples were run with brain spectrin and avidin-ferritin in
the absence of fragments. Samples were diluted to 10^-8 M of brain
spectrin in 0.1 M ammonium formate, 30% (v/v) glyceral, 1 mM NaBr,
pH 7, and within 2 min sprayed onto freshly cleaved mica. The mica
sheets were dried under vacuum at 24 °C and rotary-shadowed with
platinum at an angle of 6° followed by carbon (Fowler and Erickson,
1979; Shotton et al., 1979). Fields are shown of brain spectrin and
avidin-ferritin in the presence (A) and absence (B) of biotin-labeled
brain fragment. Selected molecules are shown below at higher mag-
nification. See Table II for quantitation of the per cent of spectrin
molecules labeled and location of the label in the middle region.

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for a nonerythroid ankyrin are as follows: 1) cross-reactivity with erythrocyte ankyrin; 2) $M_\text{T}$ of $\sim 200,000$; 3) association with membranes; 4) protease-resistant domains of $M_\text{T} = 95,000$ and $72,000$; 5) binding of the $72,000 M_\text{T}$ domain to spectrin; 6) localization of the binding site on spectrin to the midregion of spectrin tetramers; 7) presence in approximately equivalent amounts as spectrin tetramer. Microtubule-associated proteins of $M_\text{T} = 370,000$ have been identified in brain that cross-react with ankyrin (Bennett and Davis, 1981; Bennett et al., 1982b). The microtubule-associated proteins appear to be not as closely related to ankyrin in terms of antigenic sites or in other structural and functional features. The microtubule-associated proteins should be viewed as perhaps related to ankyrin in terms of a common evolutionary origin, but presently as a distinct group of proteins.

The membrane-associated ankyrin proteins are similar enough to erythrocyte ankyrin to be considered members of a common family of proteins. It is likely that additional isoforms of ankyrin will be discovered in different tissues or during differentiation of the same cell, by analogy with various forms of brain and muscle spectrin (Nelson and Lazarides, 1983). Nomenclature may become complicated, but at the present time these proteins can be referred to based on the tissue or cell of origin, e.g. hepatocyte ankyrin, brain ankyrin, etc.

Brain spectrin and ankyrin are not identical to their analogues in erythrocytes. One difference already evident is that the affinity of ankyrin-spectrin binding is lower for brain proteins, with a $K_d$ of $0.5-1 \mu M$ rather than a $K_d$ of $0.02-0.05 \mu M$ observed for erythrocyte proteins (not shown). The average concentration of brain ankyrin and spectrin is about $1 \mu M$ in brain tissue (estimated on the basis of $30 \text{ pmol/mg}$ of membrane protein, $30 \text{ mg}$ of membrane protein/g of tissue), and local concentrations of spectrin and ankyrin on membrane surfaces are most likely 10-20 fold higher. Thus, the concentrations of spectrin and ankyrin on the membrane are sufficiently high for a major portion of these proteins to exist as a spectrin-ankyrin complex. However, the relatively low affinity compared to erythrocyte membranes suggests that the spectrin-ankyrin associations may be more dynamic than in erythrocytes and may be subject to regulation. Another important difference between membranes from brain and mammalian erythrocytes is that brain membranes contain tubulin (Bhattacharya and Wolff, 1975) and most likely also have binding sites for tubulin (Bernier-Valentin et al., 1983). Erythrocyte ankyrin binds to microtubules assembled from pure brain tubulin (Bennett and Davis, 1981), and it is possible that brain ankyrin also has a binding site for microtubules and is complexed with tubulin on the membrane.

Ankyrin is the major membrane attachment site for spectrin in erythrocytes, but in brain and other tissues, spectrin may have additional membrane linkages. Preliminary measurements of association of $^{125}$I-labeled spectrin to brain membranes indicated saturable, high affinity binding that persisted after extraction of brain $72,000 M_\text{T}$ fragment and was not displaced by fragment (unpublished data). Furthermore, spectrin-binding polypeptides unrelated to the $72,000 M_\text{T}$ fragment were recovered from the spectrin affinity column during purification of the fragment (Fig. 5). In fact, there presently is no direct evidence that brain ankyrin links spectrin to the membrane, although all of the available data is consistent with such an association. Elucidation of the membrane associations of brain spectrin clearly will be more complex than the studies in erythrocytes, and will be the subject of future work.

An important extension of the present studies will be to determine the protein(s) that links brain ankyrin to the membrane. If the analogy with erythrocyte membranes continues to be relevant, then the ankyrin-binding protein will be an integral membrane protein and also contain an ion channel. This putative protein may also associate with other integral proteins, as is the case with the erythrocyte anion channel and other membrane proteins in erythrocytes. The existence of an integral membrane protein or family of such proteins capable of lateral associations in the membrane and of binding to cytoskeletal proteins could explain how membrane proteins are restricted in their motion and localized at specialized regions on the cell surface.

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Supplemental Information

3 Binding of fragment and brain spectrin was measured with brain fragment labeled with $^{125}$I-Bolton Hunter reagent; spectrin-bound fragment was separated from free fragment by immunoprecipitation with antibody against spectrin as described (Bennett and Stenbuck, 1980).
Brain ankyrin. Purification of a 72,000 Mr spectrin-binding domain.
J Q Davis and V Bennett


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