Brain Ankyrin

PURIFICATION OF A 72,000 Mₐ 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. Erythrocyte ankyrin M₂ 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 hydroxyapatite. 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 radioimmunoassay with ¹²⁵I-labeled brain fragment and antibody against erythrocyte ankyrin. 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 at 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.

Analogs 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 radioimmunoassay 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 spectrins. Brain ankyrin is present in approximately the same quantities as brain spectrin tetramers and is a logical candiate 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—Na¹⁹⁸I was from Amersham. Hydroxyapatite (fast flow), Protein A-bearing staphylococci, and biotin-N-hydroxysucc-
cinoglonate-activated Sepharose 4B, Protein A, Protein A-Sepharose, and Sephacyr-S 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 M₉ was purified from pig and human erythrocytes as described (Bennett, 1978). 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 E₂₈₀ of brain ankyrin 72,000 M₉ was 9.0 based on protein determination by Bradford assay, and λ₃₅₀ was used to estimate protein with purified preparations of enzyme. Autoradiography was performed with X-Omat AR film (Kodak) and Cronex intensifier screens (DuPont). Proteins were radioiodinated with Na₁₂⁵¹ 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, 2 mM sodium EGTA, 10 mM Na₂SO₄, 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 mg/ml of antibody. The nitrocellulose paper was washed 5 times with buffer (no albumin) and once with 2 M urea, 0.1 M glycine, 1% Triton X-100. The bound antibody was labeled by incubation 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 cyanoglonate-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 M NaN₃. The gel was then washed with 10 liters of 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 M₉ = 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 M₉ = 220,000 and many polypeptides of lower M₉. Brain membranes contain two major cross-reacting polypeptides of M₉ = 220,000 and 215,000, and a minor polypeptide of M₉ = 190,000. The relative amounts of the M₉ = 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 M₉ = 215,000 and 190,000 polypeptides may be proteolytic products of the M₉ = 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 M₉ = 190,000 and a fainter cross-reacting band at M₉ = 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, PMSF, 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 M₉ = 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 ($M_r = 260,000$ 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 in 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 entracted the major portion of the 95,000 $M_r$ fragment while the 72,000 $M_r$ fragment was extracted from the digested membranes with 0.5 M NaCl (lane 4), supernatant after digestion with $\alpha$-chymotrypsin (lane 5), supernatant after extraction with 0.5 M NaCl (lane 6). Proteins in a parallel gel were transferred electrophoretically to nitrocellulose, and polypeptides cross-reacting with erythrocyte ankyrin were visualized (see "Experimental Procedures") (right).

**FIG. 2.** Subcellular distribution of brain polypeptides cross-reacting with erythrocyte ankyrin. Frozen pig brain was homogenized (see "Experimental Procedures") in ten volumes of 0.32 M sucrose, 2 mM sodium EGTA, 200 $\mu$g/ml of PMSF, 0.015% (v/v) DFP, 5 $\mu$g/ml of leupeptin, and 2 $\mu$g/ml of pepstatin A, and centrifuged at 900 $\times$ g for 10 min. The pellets from centrifugation of the 900 $\times$ g supernatant at 30,000 $\times$ g, and 200,000 $\times$ g, respectively, were resuspended to the original volume of homogenization buffer. The resuspended 30,000 $\times$ g pellet was layered over a 13-ml linear gradient of 15-60% sucrose dissolved in 2 mM sodium EGTA, 100 $\mu$g/ml of PMSF, pH 7.5, and centrifuged 4 h at 40,000 $\times$ g in a SW-41 rotor. Fractions containing the dense membrane peak (40-50% sucrose) and the lighter myelin membranes were pooled. The samples were then extracted twice (30 min, 4 °C) in the same buffer with 0.5 M NaCl. The membranes were resuspended in buffer without salt or PMSF and digested with 20 $\mu$g/ml of $\alpha$-chymotrypsin for 1 h at 4 °C. The digestion was stopped by addition of DFP (0.015%) and PMSF (200 $\mu$g/ml) and the membranes pelleted. The 72,000 $M_r$ fragment was then extracted with 0.5 M NaCl, 10 mM sodium phosphate, 0.5 mM EDTA, 0.5 mM DTT. Samples were dissolved in SDS and electrophoresed on an SDS-polyacrylamide gel (left): 900 $\times$ g supernatant (lane 1), 30,000 $\times$ g pellet (lane 2), 200,000 $\times$ g pellet (lane 3), 200,000 $\times$ g supernatant (lane 4), myelin fraction (lane 5), dense membrane fraction (lane 6). Proteins in a parallel gel were transferred electrophoretically to nitrocellulose, and polypeptides cross-reacting with erythrocyte ankyrin were visualized (see "Experimental Procedures") (right).

**FIG. 3.** Extraction of a 72,000 $M_r$, polypeptide cross-reacting with erythrocyte ankyrin following digestion of brain membranes with $\alpha$-chymotrypsin. Brain membranes were processed in the same fashion as described in Fig. 5 except that all postnuclear centrifugation was performed in a 60 Ti rotor (50,000 rpm, 10 min). Briefly, membranes were washed first in 10 mM sodium phosphate, 1 mM sodium EGTA, 50 $\mu$g/ml of PMSF, pH 7.5, and then extracted twice (30 min, 4 °C) in the same buffer with 0.5 M NaCl. The membranes were resuspended in buffer without salt or PMSF and digested with 20 $\mu$g/ml of $\alpha$-chymotrypsin for 1 h at 4 °C. The digestion was stopped by addition of DFP (0.015%) and PMSF (200 $\mu$g/ml) and the membranes pelleted. The 72,000 $M_r$, fragment was then extracted with 0.5 M NaCl, 10 mM sodium phosphate, 1 mM Na EGTA, 0.5 mM DTT. Samples were dissolved in SDS and electrophoresed on an SDS-polyacrylamide gel stained with Coomassie blue (left): starting membranes (lane 1), membranes after first extraction with 0.5 M NaCl (lane 2), supernatant after extraction with 0.5 M NaCl (lane 3), membranes after second extraction with 0.5 M NaCl (lane 4), membranes after digestion with $\alpha$-chymotrypsin (lane 5), supernatant after digestion with $\alpha$-chymotrypsin (lane 6), digested membranes after extraction with 0.5 M NaCl (lane 7), supernatant after extraction of digested membranes with 0.5 M NaCl (lane 8). Proteins in a parallel gel were transferred electrophoretically to nitrocellulose, and polypeptides cross-reacting with erythrocyte ankyrin were detected (see "Experimental Procedures").

since limited digestion with $\alpha$-chymotrypsin degraded the $M_r = 220,000, 215,000$, 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 hydroxylapatite (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.
Figure 4. Binding of brain 72,000 M<sub>r</sub> ankyrin fragment to an erythrocyte spectrin affinity column, and binding of 125I-labeled erythrocyte spectrin to brain 72,000 M<sub>r</sub> fragment transferred to nitrocellulose from an SDS-polyacrylamide gel. Brain ankyrin 72,000 M<sub>r</sub>, fragment was extracted from 200 g of membranes, and precipitated with ammonium sulfate as in Fig. 5. The fragment was partially purified by DEAE-chromatography and eluted with a gradient of 10-300 mM NaCl at pH 7.5. The peak containing 72,000 M<sub>r</sub> fragment was identified by immunoblots (see "Experimental Procedures"), and applied to an erythrocyte spectrin-affinity column (12 ml gel; 1.5 mg of pig erythrocyte spectrin/ml of Sepharose 4B (see "Experimental Procedures") equilibrated with 0.1 M NaCl, 10 mM sodium phosphate, 1 mM sodium azide, 0.5 mM DTT. Fractions from the affinity column were monitored by A<sub>280</sub> and by immunoblotting to detect polypeptides cross-reacting with erythrocyte ankyrin. The affinity column was eluted with 400 ml of 0.5 M sodium bromide, 10 mM sodium phosphate, 1 mM sodium azide, 0.2 mM DTT directly onto a 2-ml hydroxylapatite column which adsorbed brain 72,000 M<sub>r</sub>, fragment. The fragment was recovered from the hydroxylapatite column by eluting with 0.4 M sodium phosphate, pH 7.3. Fractions were electrophoresed on an SDS-polyacrylamide gel stained with Coomassie blue (left panel); erythrocyte ankyrin 72,000 M<sub>r</sub>, fragment (lane 1), affinity column starting material (lane 2), affinity column breakthrough (lane 3), hydroxylapatite eluate (lane 4). Proteins were electrophoresed on a parallel gel and transferred electrophoretically to nitrocellulose paper for detection of polypeptides cross-reacting with erythrocyte ankyrin (see "Experimental Procedures" and Fig. 1) (second panel) and for binding of 125I-labeled erythrocyte spectrin (right three panels). Binding of erythrocyte spectrin was achieved by incubation of the nitrocellulose paper with transferred polypeptides 14 h at 4 °C in immunoblot buffer (see "Experimental Procedures") with 12 nM 125I-labeled erythrocyte spectrin (8 × 10<sup>5</sup> cpn/pmol) alone or with additions of either 200 nM unlabeled erythrocyte spectrin, or 100 nM erythrocyte ankyrin 72,000 M<sub>r</sub>, fragment. The nitrocellulose was then washed five times with immunoblot buffer lacking bovine serum albumin and dried, and an autoradiogram was prepared.

The purified 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.
For explanation, see Fig. 5.

Table I

| Fraction*                      | Protein  |
|--------------------------------|----------|
| Washed membranes               | 7290.0   |
| 0.5 M NaCl pre-extracted membranes | 5700.0   |
| 0.5 M NaCl pre-extract         | 1554.0   |
| Post chymotrypsin membranes    | 5431.0   |
| Chymotrypsin supernatant       | 585.0    |
| 0.5 M NaCl extracted membranes | 5335.0   |
| 0.5 M NaCl extract             | 163.0    |
| Ammonium sulfate insoluble     | 19.0     |
| DE53 0.5 M NaCl cut            | 46.0     |
| Spectrin-Sepharose breakthrough| 6.0      |
| Purified brain fragment        | 0.3      |

*For explanation, see Fig. 5.

Table II

| Location of ferritin | Biotin-labeled brain fragment (1078 molecules counted) | Biotin-labeled red blood cell fragment (546 molecules counted) | Control (981 molecules counted) |
|----------------------|------------------------------------------------------|------------------------------------------------------------|---------------------------------|
| Middle               | 16.2                                                 | 13.2                                                       | 6.1                             |
| End                  | 6.2                                                  | 4.7                                                        | 5.5                             |
| Other                | 6.1                                                  | 6.7                                                        | 3.9                             |

% bound minus controls

Middle | 10.1 | 7.1 |
End   | 0.7  | 3.7 |
A major concern was that the 72,000 M, 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 M, 130,000 cross-reacting with brain spectrin, 72,000 M, ankyrin fragment, and a polypeptide at 43,000 M, that may be actin. The fragment was purified about 400-fold with a yield of only 300 pmol 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 M, fragment was observed 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 M, fragment to antibody against erythrocyte 72,000 M, fragment by rat erythrocytes and rat brain membranes.

Localization of the Binding Site on Brain Spectrin for Brain and Erythrocyte 72,000 M, 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 1:1:1 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-

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**Fig. 6.** Two-dimensional maps of 125I-labeled chymotrypsin peptides of pig erythrocyte ankyrin 72,000 M, fragment (A), brain ankyrin 72,000 M, fragment (B), and a mixture of peptides from both erythrocyte and brain fragments (C). Pig erythrocyte 72,000 M, fragment and brain 72,000 M, 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/pmol. The incubation was continued for.

plexes were pelleted.

ends and 3-fold more labeling than all other sites on the spectrin tetramer, and in 6% of the molecules at

the ends of spectrin tetramers, and in 6% of the molecules at

other regions along spectrin molecules.

Ferritin-labeling of the midregion of spectrin incubated

at each concentration of protein tested.

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 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 polypeptides of \( M_r = 190,000-220,000 \) in brain, lens, and liver that cross-react with erythrocyte ankyrin. The cross-reacting polypeptides in brain, and most likely other tissues as well, are closely related to erythrocyte ankyrin in structure and function. The brain polypeptides 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 \( M_r \) fragment binds to erythrocyte spectrin at the same site as erythrocyte 72,000 \( M_r \) fragment (Fig. 4) and was purified by affinity chromatography on erythrocyte spectrin-agarose (Fig. 5). Purified brain 72,000 \( M_r \) fragment was distinct from erythrocyte 72,000 \( M_r \) fragment 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 \( M_r \) fragment was estimated by radioimmunoassay 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 widespread presence of proteins closely related to ankyrin in cell membranes. The criteria that seem appropriate at this point.
for a nonerythroid ankyrin are as follows: 1) cross-reactivity with erythrocyte ankyrin; 2) Mr of ~200,000; 3) association with membranes; 4) protease-resistant domains of Mr = 95,000 and 72,000; 5) binding of the 72,000 Mr 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 Mr = 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 not be 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 Kd of 0.5–1 μM rather than a Kd of 0.02–0.05 μM observed for erythrocyte proteins2 (not shown). The average concentration of brain ankyrin and spectrin is about 1 μM in brain tissue (estimated on the basis of 30 pmol/mg of membrane protein, 30 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 (Bhattacharyya 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 125I-labeled spectrin to brain membranes indicated saturable, high affinity binding that persisted after extraction of brain 72,000 Mr fragment and was not displaced by fragment (unpublished data). Furthermore, spectrin-binding polypeptides unrelated to the 72,000 Mr 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 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 to specialized regions on the cell surface.

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3 Binding of fragment and brain spectrin was measured with brain fragment labeled with 125I-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.
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