Brain Spectrin(240/235) and Brain Spectrin(240/235E): Two Distinct Spectrin Subtypes with Different Locations within Mammalian Neural Cells

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Abstract. Adult mouse brain contains at least two distinct spectrin subtypes, both consisting of 240-kD and 235-kD subunits. Brain spectrin(240/235) is found in neuronal axons, but not dendrites, when immunohistochemistry is performed with antibody raised against brain spectrin isolated from enriched synaptic/axonal membranes. A second spectrin subtype, brain spectrin(240/235E), is exclusively recognized by red blood cell spectrin antibody. Brain spectrin(240/235E) is confined to neuronal cell bodies and dendrites, and some glial cells, but is not present in axons or presynaptic terminals.

SINCE the discovery of nonerythroid spectrins (Goodman et al., 1981; Glenney et al., 1982; Bennett et al., 1982; Repasky et al., 1982; Burridge et al., 1982), the brain analogue has become the most completely described member of this family of proteins. Brain spectrin is a 1,000,000-mol-wt fibrous protein, with subunits of 240 kD (α) and 235 kD (β) forming an $(\alpha\beta)_2$ tetrameric complex. The α subunit contains a binding site for calmodulin, while the phosphorylated β subunit contains a binding site for brain syndein/ankyrin, a potential membrane attachment site. Brain protein 4.1 and f-actin attach to both ends of the bivalent brain spectrin tetramer (for reviews see Glenney and Glenney, 1983b; Goodman and Zagon, 1984; Bennett, 1985; Goodman and Zagon, 1986). Despite the rapid expansion of our knowledge concerning the structure and protein interactions of brain spectrin over the past four years, we do not as yet have an accurate picture of the precise intracellular location of spectrin within neural cells. This informational gap has been limiting our ability to ask appropriate questions concerning the potential functions of spectrin within nerve cells. Attempts to localize spectrin in mammalian nervous tissue by immunohistochemical analysis have yielded a perplexing set of facts, which on first glance appear contradictory.

Levine and Willard prepared antibodies against an axonally transported protein with subunits of 240 kD and 235 kD, which they named fodrin (Levine and Willard, 1981). Fodrin was isolated by high salt extraction of guinea pig crude brain membranes followed by gel filtration and preparative gel electrophoresis (Levine and Willard, 1981), and the antibody raised in rabbits cross-reacted primarily with the 240-kD α subunit of the 240-kD/235kD protein doublet (Cheney et al., 1983). When indirect immunofluorescence studies were performed, fodrin antibodies stained the periphery of guinea pig neuronal cell bodies, dendrites and axons in the peripheral nervous system, as well as staining the plasma membrane of Schwann cells (Levine and Willard, 1981). We now know that fodrin is a member of the nonerythroid spectrin family of molecules and refer to it as brain spectrin (for review see Goodman and Zagon, 1984). Our laboratory performed a systematic study of the distribution of spectrin in the central nervous system of mammals, using sagittal and coronal sections of the mouse brain and an antibody against mouse red blood cell (rbc)[1] spectrin (Zagon et al., 1984). As our antibody stained both 240-kD and 235-kD polypeptides co-migrating with the brain spectrin α and β subunits on immunoautoradiography of total mouse brain homogenate protein separated by SDS PAGE, we expected to see the intense staining of neuronal perikarya, dendrites, and axons described in Willard's elegant study (Levine and Willard, 1981). Instead, immunofluorescent studies using monospecific mouse rbc spectrin antibody consistently revealed staining of all neuronal cell bodies and dendrites, but no staining of axons, synaptic structures, or nuclei (Zagon et al., 1984). After eliminating species differences, and differences between spectrin in central versus peripheral nervous system as the basis for these apparent contradictions in data (Goodman and Zagon, 1984), we were faced with the following conundrum. If antibodies against mammalian brain spectrin and rbc spectrin both specifically detect brain spectrin on immunoautoradiographic analysis of total brain protein, then why do brain spectrin antibodies stain perikarya, dendrites, and axons, while rbc spectrin antibodies stain only perikarya and dendrites on immunohistochemical analysis?

1. *Abbreviations used in this paper:* FCS, fetal calf serum; MAP, microtubule-associated protein; RBC, red blood cell; TBS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl; TBSA, 5% BSA in TBS.

We demonstrate here, that mammalian brain contains at least two distinct spectrin subtypes both of which have subunits of 240 and 235 kD. Brain spectrin(240/235) (nomenclature of Goodman and Zagon, 1984) is located in neuronal axons and, to a lesser extent, cell bodies. This brain spectrin subtype cannot be detected with rbc spectrin antibodies. Brain spectrin(240/235E) (E stands for erythrocyte subtype) is found in neuronal cell bodies and dendrites, but not in axons. This brain spectrin subtype is recognized specifically by an antibody directed against rbc spectrin. These studies reconcile all previous observations on the location of spectrin in mammalian brain, and suggest that a new level of sophistication in our approaches to mammalian brain spectrin function is appropriate. Two subtypes of spectrin have previously been described in avian brain, chicken brain spectrin(240/235), and brain spectrin (240/230) (Lazarides and Nelson, 1983a, b). Similarities and differences between the avian and mammalian brain spectrin subtypes are discussed.

Materials and Methods

Isolation of Spectrins

Forty mice were anesthetized with ether, and after transcardiac perfusion with phosphate-buffered saline (PBS), pH 7.4, with 1% sodium citrate, the perfused blood was collected and kept on ice. Brains were removed and immediately frozen in liquid nitrogen. Starting with blood perfusate of 40 mice, rbc ghosts were prepared as described for human rbc ghosts (Goodman et al., 1982b) and rbc spectrin was purified by the method of Goodman et al. (1982a).

Brain Spectrin Isolation. Brain spectrin(240/235) was isolated from the P_2B fraction of mouse brain homogenates prepared with only minor modifications of the procedure of Gray and Whittaker (1962). 15 g frozen mouse brain tissue was homogenized in 100 ml 0.32 M sucrose, 1 mM EDTA, 10 mM Na_2PO_4, pH 7.0, 400 µM diisopropylfluorophosphate. The homogenate was centrifuged at 900 g (5 min, 2°C), and the supernatant sedimented at 39,000 g (20 min, 2°C). The crude synaptosomes were resuspended in wash buffer (10 mM Na_2PO_4, pH 7.0, 1 mM EGTA, 400 µM diisopropylfluorophosphate) to a final volume of 40 ml and loaded onto four 28-ml sucrose step gradients of 1.2 and 0.8 M sucrose in the same buffer. Gradients were centrifuged at 106,000 g (65 min, 2°C). The P_2B fraction from the 0.8/1.2 M sucrose interphase was washed in 3 vol of wash buffer and centrifuged at 39,000 g (20 min, 2°C). Extraction of spectrin from the P_2B fraction was performed by a modification of previously described procedures (Bennett et al., 1982; Goodman et al., 1983). The pellet was resuspended in 100 ml ice cold low ionic strength buffer (0.1 mM EGTA, 0.25 mM dithiothreitol, 200 µM diisopropylfluorophosphate, pH 7.0) and centrifuged at 39,000 g (20 min, 2°C). Crude spectrin was extracted from the pellet by an incubation with 100 ml low ionic strength buffer at 37°C. The suspension was centrifuged at 200,000 g (30 min, 2°C). Proteins were precipitated by adding 0.35 g ammonium sulfate/ml supernatant at pH 7.0, and kept on ice overnight. After a 15,000 g centrifugation (20 min, 2°C), the ammonium sulfate precipitate was dissolved in 3 ml glycine–urea buffer (25 mM glycine, 1 mM EGTA, 1 mM NaN₃, 0.25 dithiothreitol, 1 M urea, pH 8.0) and dialyzed for 4 h at 4°C against the same buffer. The extract was centrifuged at 200,000 g (3 h, 2°C). The supernatant was overlayed onto 5–18% linear sucrose gradients in glycine–urea buffer. The larger spectrin complexes were separated from smaller proteins by rate zonal sedimentation at 286,000 g (18 h, 2°C). Fifteen 0.8-ml fractions were collected from the bottom of the tubes. Fractions 5–10 were loaded onto a 70-ml Sepharose 4B column, equilibrated with PBS. Pure brain spectrin(240/235) was eluted immediately after the void volume of the column.

Immunological Methods

Antibody Production. Native homogeneous mouse brain spectrin(240/235) and rbc spectrin were used as immunogens for raising antibodies in rabbits as described (Goodman et al., 1981). The rbc spectrin antibody was previously characterized (Goodman et al., 1984b; Zagon et al., 1984). For the quantitative characterization of the antisera the immunodot assay of Hawkes et al. (1982) and Jahn et al. (1984) were modified. Protein solutions (0.5 µl) were spotted onto nitrocellulose paper with preprinted 3 × 3-mm grids (Millipore/Continental Water Systems, Bedford, MA). The dried filters were washed with 50 mM

Tris-HCl, pH 7.4, 150 mM NaCl (TBS) and blocked for 1 h in 5% bovine serum albumin in TBS (TBSA). The filters were incubated overnight with antiserum diluted 1:500 in TBSA and 0.1% Triton X-100. The dots were rinsed four times (5 min) with TBSA and blocked for 30 min with TBSA. The filters were incubated for 2 h with ¹²⁵I-protein A (0.1 µCi/ml) in TBSA and 0.1% Triton X-100, washed four times (5 min) with TBS and 0.1% Triton X-100, and four times (20 min) with TBS. The wet filters were cut along the grids and assayed for radioactivity in a Packard 500 gamma counter. Each determination was performed in triplicate. All steps were done at room temperature. The same procedure was also used for staining certain immunoblots, and autoradiography of the dry immunoblots was performed using Kodak X-Omat XAR5 film exposed at −20°C.

Antisera Specificity. 8 mg brain spectrin(240/235) and 3 mg rbc spectrin were coupled to 1 g CNBr-activated Sepharose 4B by the manufacturer's recommended procedure (Pharmacia Fine Chemicals, Piscataway, NJ). The coupling efficiency tested by the dot assay was >90%. The spectrin–Sepharose 4B columns were pretreated with 2 M $MgCl_2$ in PBS and washed with PBS. The antisera against rbc and brain spectrin(240/235) were passed through the brain and rbc spectrin–Sepharose 4B column, respectively. The recovery, specificity, and cross-reactivity of the cleaned antisera was tested by the quantitative dot assay and immunoblots. The spectrin–Sepharose 4B columns were restored by 2 M $MgCl_2$ and PBS wash cycles. The antisera were passed through the columns at least three times. The IgG content of the sera was determined by a quantitative dot assay using defined amounts of rabbit IgG (Sigma Chemical Co., St. Louis, MO) as standards. To test the specificity of the antisera, proteins were transferred from polyacrylamide SDS slab gels to nitrocellulose filter (Millipore/Continental Water Systems) by the method of Towbin et al. (1979). The transfer buffer contained additional 0.005% SDS. A satisfying transfer was achieved after 6 h at 7 V/cm at 4°C. The proteins on the filter were either stained with Amido black or antiserum. For immunological staining of the gel blots the procedure for the dot assay of Hawkes et al. (1982) was adapted and modified. Blots were washed with TBS for 5 min, blocked for 15 min with 10% normal horse serum in TBS, and incubated overnight with antisera diluted 1:1,000 in the blocking solution with 0.1% Triton X-100. After four wash cycles with TBS, the filters were incubated briefly in blocking solution alone, and 3 h in blocking solution together with 0.1% horseradish peroxidase–conjugated goat anti-rabbit immunoglobulins (Cappel Laboratories, Cochranville, PA) and 0.1% Triton X-100. The blots were washed by four cycles with TBS. The peroxidase reaction was developed in 0.06% 4-chloro-1-naphthol, 0.01% hydrogen peroxide in TBS for 15 min. The filters were washed and air dried.

Immunohistochemistry

Freshly perfused mouse brains were frozen with freon. The 10-µm thick cryostat sections were treated with 95% ethanol (30 min) and acetone (20 min) at 4°C. The sections on coverslips were washed with TBS for 5 min at room temperature and blocked with 3% fetal calf serum (FCS) in TBS for 15 min. The sections were incubated overnight with antiserum diluted 1:100 in 1% FCS in TBS with 0.1% Triton X-100. Unbound antibodies were washed away with four washes of 1% FCS, 0.1% Triton X-100 in TBS and the sections were blocked again with 3% FCS in TBS for 15 min, followed by a 3-h incubation with 0.3% horseradish peroxidase–conjugated goat anti-rabbit IgG in 1% FCS, 0.1% Triton X-100 in TBS. Excess peroxidase was washed away with four changes of TBS over 30 min, and the remaining peroxidase activity was stained with 0.06% 4-chloro-1-naphthol, 0.01% hydrogen peroxide in TBS for 15 min. The sections were washed with TBS and mounted onto slides with 9:1 glycerol/TBS.

Electrophoresis

SDS PAGE. Protein samples were separated on 5–15% acrylamide SDS slab gels (Laemmli, 1970). Gels were stained with 0.025% Coomassie Brilliant Blue R in 25% isopropanol, 10% acetic acid overnight, and destained with 10% isopropanol, 10% acetic acid.

Results

Spectrin Isolation from Synaptic Membranes

Rather than isolating spectrin from crude brain homogenates (Levine and Willard, 1981; Glenney et al., 1982; Burridge et al., 1982; Bennett et al., 1982; Goodman et al., 1983, 1984b), we first prepared a synaptic/axonal membrane fraction by the

standard protocol of Gray and Whittaker (1962). The P_2 fraction of mouse brain homogenate was further fractionated on a sucrose step gradient, and the material collected from the 0.8–1.2 M sucrose interphase (referred to as the P_2B fraction by Gray and Whittaker, 1962), was used for subsequent spectrin isolations. The P_2B fraction has previously been demonstrated to be enriched in synaptic vesicles, axon segments and, to a lesser extent mitochondria (Gray and Whittaker, 1962); we will refer to it simply as an enriched synaptic and axonal membrane preparation. As demonstrated in Fig. 1, we have isolated brain spectrin(240/235) by low ionic strength extraction of the P_2B fraction, followed by rate zonal sedimentation through a linear 5–18% sucrose gradient, and gel filtration chromatography on a Sepharose 4B column. Highly purified brain spectrin(240/235) was eluted from the column immediately after the void volume. The yield of brain spectrin(240/235) from the P_2B fraction was 0.22 mg brain spectrin/g mouse brain tissue, a value which is four- to eightfold greater than previous isolations from crude brain homogenates. In addition to the higher yield of spectrin isolated from the P_2B fraction, we reasoned that if a synaptic/axonal form of spectrin existed in mammalian neurons we would be enriching this spectrin subtype of beginning the preparation with the P_2B fraction rather than total brain protein.

Characterization of Antisera

Antiserum raised in rabbits against native mouse brain spectrin(240/235) and mouse rbc spectrin were tested for affinity and specificity by a quantitative immunodot assay (Fig. 2). The antiserum against mouse brain spectrin(240/235) was capable of detecting purified brain spectrin(240/235) down to ~1.5 ng, but also cross-reacted with purified mouse rbc spectrin with a sensitivity to 5 ng (Fig. 2A). We wanted to have a probe which would strongly detect brain spectrin(240/235),

but would not be very sensitive to any rbc-related spectrin subtype, if such a subtype existed in mammalian brain. We therefore passed the brain spectrin(240/235) antiserum through an rbc spectrin–Sepharose 4B affinity column six times in an attempt to remove antibody capable of detecting rbc spectrin or rbc spectrin subtypes in brain. As shown in Fig. 2B, this approach was successful as the depleted antiserum against brain spectrin(240/235) was still sensitive to 1.5 ng of purified brain spectrin(240/235), but showed little cross-reactivity with rbc spectrin (detection to ~50 ng). The antiserum against mouse rbc spectrin which has previously been described (Zagon et al., 1984), is extremely sensitive in detecting mouse rbc spectrin to 0.5 ng (Fig. 2C). This antibody showed very little cross-reactivity with purified brain spectrin(240/235) before or after (sensitivity to ~50 ng) three passages through a brain spectrin(240/235)–Sepharose 4B affinity column (Fig. 2D).

To demonstrate the specificity of the two antiserum before and after affinity chromatography, Western blot analysis was performed on mouse rbc membrane protein, purified mouse rbc spectrin, mouse brain homogenate protein, and purified brain spectrin(240/235) (Fig. 3). Although the antiserum against mouse brain spectrin stained the brain spectrin 240-kD α subunit, as well as the 240- and 220-kD α and β subunits of mouse rbc spectrin prior to affinity chromatography, after several passages through the rbc spectrin affinity column this antiserum recognized only the 240-kD subunit of brain spectrin(240/235) in total brain homogenates or purified brain spectrin(240/235) (Fig. 3, C and D). Therefore, through the use of the affinity chromatography depletion step, we had obtained an antibody which would recognize brain spectrin(240/235), but would not detect mouse rbc spectrin (Fig. 3D). The antiserum directed against mouse rbc spectrin stained both the α and β subunits of mouse rbc spectrin (Fig. 3, E and F). Before and after affinity chromatography through
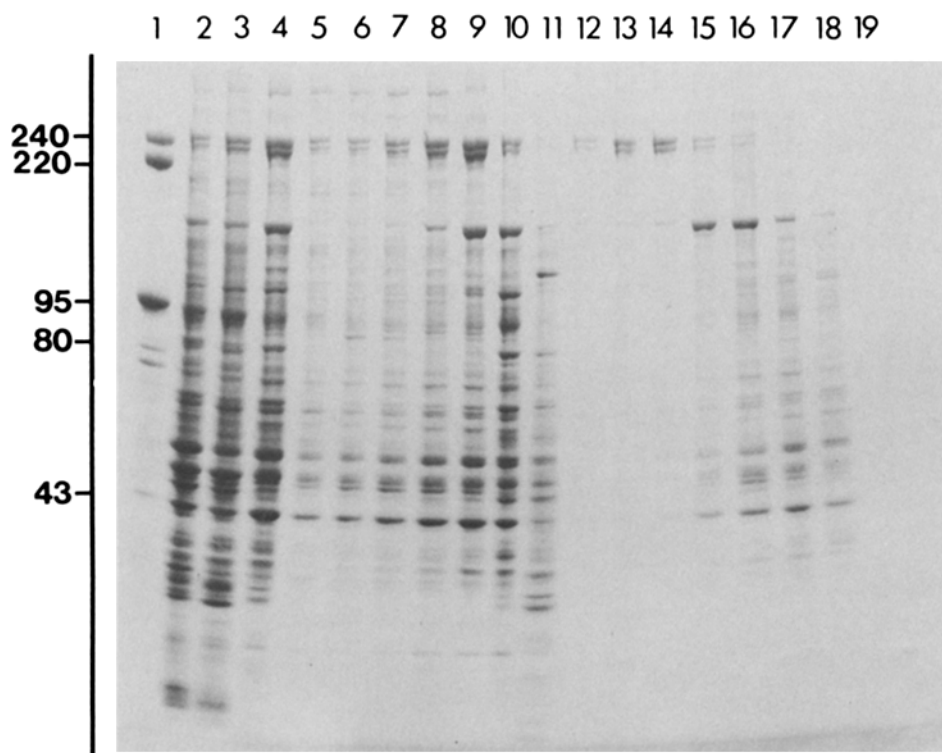

Figure 1. Brain spectrin(240/235) isolation. Coomassie Blue staining of proteins separated on 5–15% polyacrylamide slab gels. Lane *1*, mouse rbc ghost; lane *2*, brain homogenate; lane *3*, P_2B membrane fraction; lane *4*, low ionic strength buffer extract. (Lanes *5–11*) Odd numbered fractions from rate zonal sedimentation through a 5–18% sucrose gradient are presented. Lane *5* represents the bottom of the gradient. Fractions shown in lanes *7–9* were further purified on a Sepharose 4B column. (Lane *12–19*) Selected fractions from the Sepharose 4B column are presented. Lanes represent the following column fractions: (*12*) V_e/V_o = 1.15; (*13*) V_e/V_o = 1.36; (*14*) V_e/V_o = 1.56; (*15*) V_e/V_o = 1.97; (*16*) V_e/V_o = 2.18; (*17*) V_e/V_o = 2.38; (*18*) V_e/V_o = 2.58; (*19*) V_e/V_o = 2.99. Fractions collected from the Sepharose 4B column were 1.7 ml, and V_o = 25 ml.
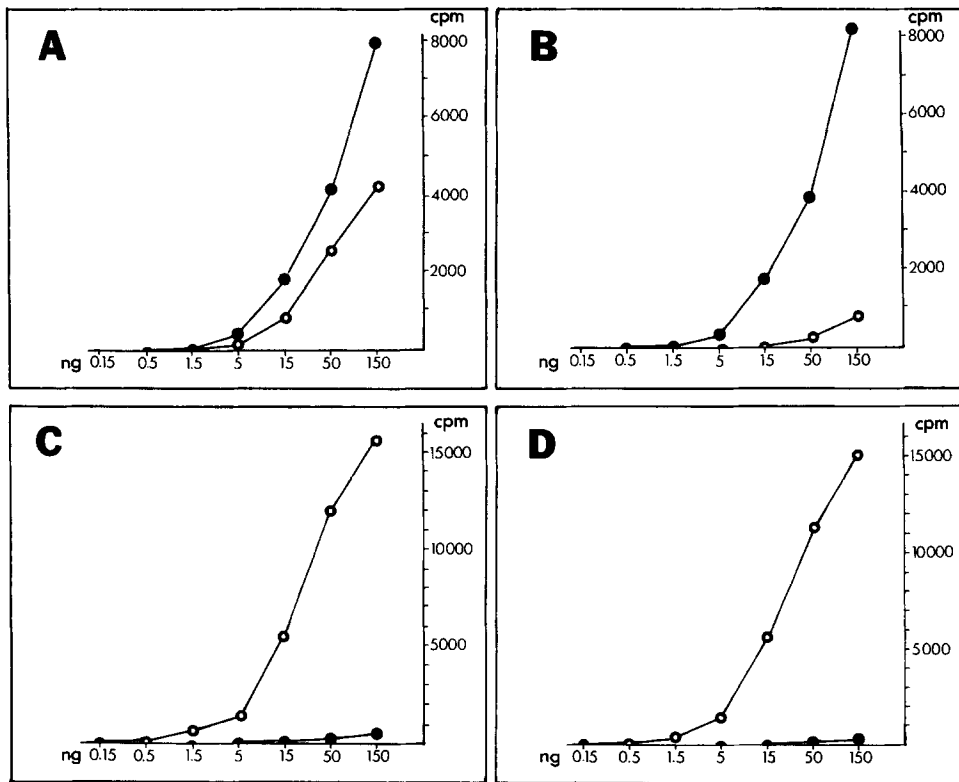
Figure 2. Quantitative dot assay with antisera against brain spectrin(240/235) (*A* and *B*) and rbc spectrin (*C* and *D*). The antisera were tested before passing through the affinity column (*A* and *C*) and after six cycles through the rbc spectrin–Sepharose 4B (*B*) and three cycles through the brain spectrin(240/235)–Sepharose 4B (*D*) column, respectively. The immunoreaction of the antisera to defined amounts (ng per dot) of brain spectrin (●) or rbc spectrin (○) is expressed in cpm. Preimmune serum gave background binding below 200 cpm. The antisera were diluted 1:500 (20 μg IgG/ml).
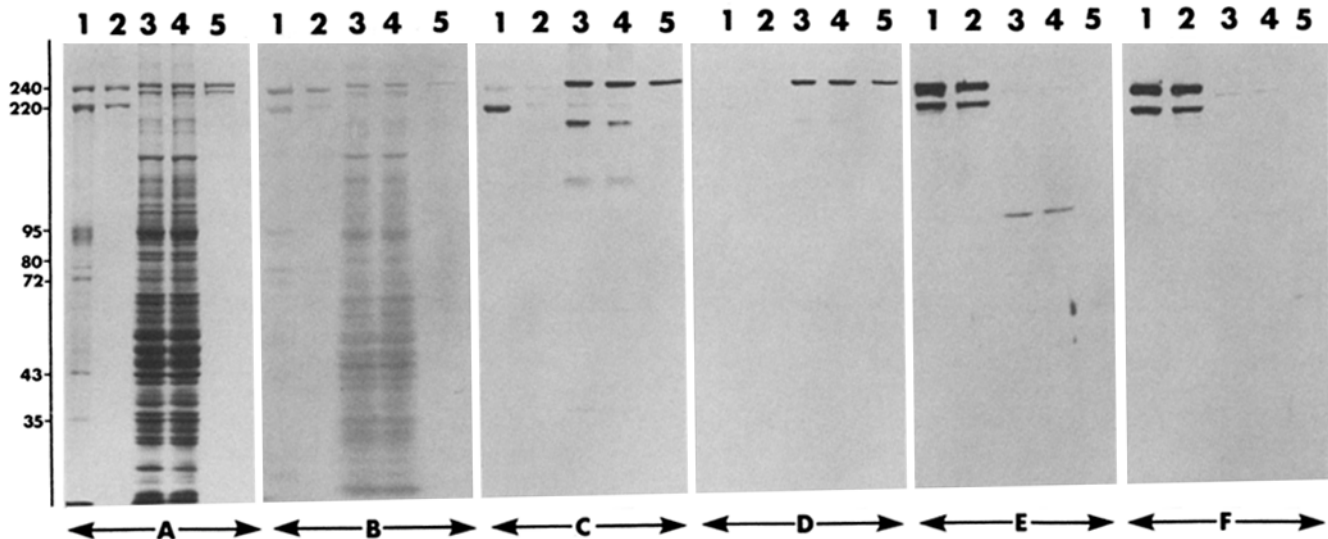
Figure 3. Specificity of the antisera. Protein samples were separated on 5–15% polyacrylamide slab gels and stained with Coomassie Blue (*A*) or transferred to nitrocellulose. Blots were stained with Amido black (*B*) or peroxidase immunostained with antiserum against brain spectrin(240/235) before (*C*) and after (*D*) passing through the rbc spectrin–Sepharose 4B column, and antisera against rbc spectrin before (*E*) and after (*F*) passing through the brain spectrin(240/235)–Sepharose 4B column. Non-immune serum showed no immunostaining of proteins. Lane *1*, mouse rbc ghost; lane *2*, pure rbc spectrin; lanes *3* and *4*, brain homogenates of two different animals; lanes *5*, pure brain spectrin(240/235) from synaptic/axonal membranes. Antisera were diluted 1:1,000 (10 μg IgG/ml).

the brain spectrin(240/235) affinity column, this antiserum did not detect purified brain spectrin(240/235) isolated from the P₂B fraction, but stained a 240- and 235-kD doublet in total brain homogenate (Fig. 3, *E* and *F*). This result which can be seen more clearly in Fig. 4*C*, suggested that mouse brain contains a 240/235-kD form of brain spectrin which can be detected by anti-mouse rbc spectrin antibody, and is

distinct from brain spectrin(240/235) which is isolated from enriched synaptic/axonal membranes and not detected by this antiserum.

To rule out the possibility that this second form of brain spectrin was the result of residual blood in the brain preparations, tissues from well perfused and nonperfused mice were compared (Fig. 4). In Fig. 4, we demonstrate that the brain
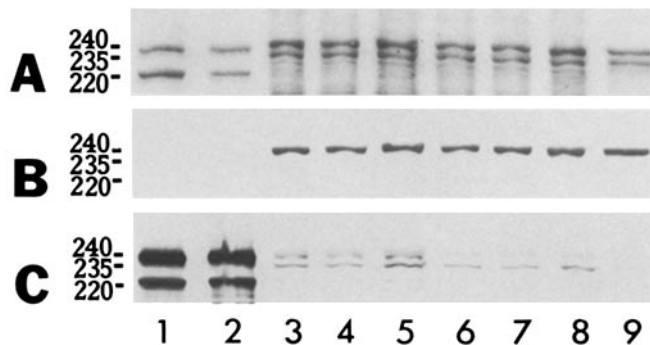
Figure 4. Subtypes of spectrin. Blots from proteins separated on 5–15% gradient polyacrylamide gels were stained with Amido black (A) or immunostained with specific antiserum against brain spectrin(240/235) (B) or rbc spectrin (C) at 1:1,000 serum dilution (10 μg IgG/ml) and a peroxidase-labeled second antibody. Protein samples were rbc ghost (lane 1), pure rbc spectrin (lane 2), total brain homogenate (lanes 3 and 6), forebrain homogenate (lanes 4 and 7), cerebellum homogenate (lanes 5 and 8), and pure brain spectrin from synaptic/axonal membranes (lane 9). Samples 3-5 were from a nonperfused mouse and samples 6-8 came from an extensively perfused mouse. Only the relevant portion of the blots are presented.

spectrin(240/235) antiserum reacts strongly with the 240-kD α subunit of brain spectrin(240/235) in total brain, forebrain, or cerebellar homogenates of perfused and nonperfused mice (Fig. 4B). This brain spectrin(240/235) antiserum specifically stains the α subunit of purified brain spectrin(240/235). The mouse rbc spectrin antiserum stains both the α and β subunits of mouse rbc spectrin, but does not detect brain spectrin(240/235) isolated from enriched synaptic/axonal membranes (Fig. 4C, lane 9). In nonperfused mouse brain, the rbc spectrin antiserum strongly detected the 240-kD and 235-kD subunits, as well as a 220-kD subunit that co-migrated with the mouse rbc spectrin β subunit (Fig. 4C, lanes 3-5). In perfused mice the staining of the 240/235kD doublet in total brain homogenates, forebrain, and cerebellum was still evident, with preferential staining of the 235-kD subunit (Fig. 4C, lanes 6-8). The lack of staining of the 220-kD subunit and diminished staining of the 240-kD subunits in perfused brain indicated that these immunoreactive bands were due to rbc contamination (compare Fig. 4C, lanes 3-5 vs. 6-8), while the remaining weak staining of the 240-kD band and the unaltered strong staining of the 235-kD subunit was due to an rbc-related subtype of brain spectrin. We refer to this subtype as brain spectrin(240/235E) (the E represents erythrocyte-related) in order to distinguish it from the brain spectrin(240/235). Similar results were obtained when antigens were detected within the polyacrylamide gel by the gel overlay method and immunoautoradiography (Kay et al., 1983) (data not shown).

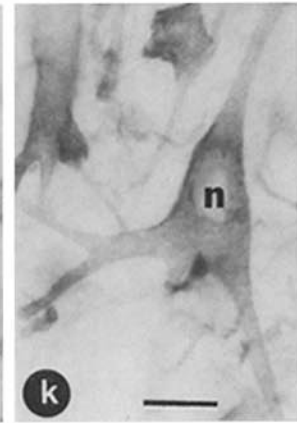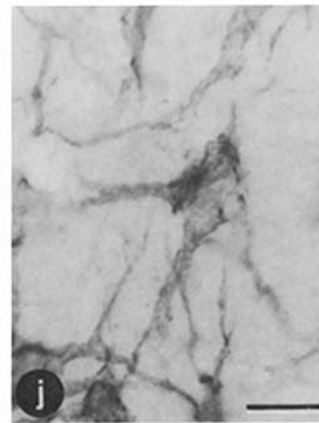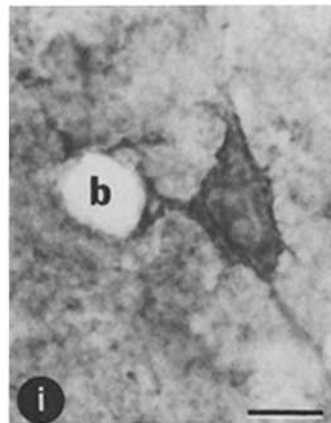## Different Distribution of Brain Spectrin(240/235) and Brain Spectrin(240/235E) in Adult Mouse Brain

Final proof that the brain spectrin(240/235) and brain spectrin(240/235E) proteins represented distinct subtypes within mammalian brain, came from extensive immunohistochemical studies which demonstrated different locales of these subtypes within neural cells (Figs. 5 and 6). Immunoperoxidase studies on sagittal cryostat sections stained first with antiserum against rbc spectrin (which specifically recognizes brain spectrin[240/235E]) or antiserum against brain spectrin(240/235) showed a strikingly different subcellular distribution of subtypes. In the cerebellum, the brain spectrin(240/235) antiserum exhibited staining of the medullary layer, the internal granule layer and molecular layer (Fig. 5a). At higher magnification, the peroxidase staining was confined to axonal processes in the medullary layer (Fig. 5, c and h), basket fibers which surround Purkinje cells (Fig. 5d), and fibers (axons) coursing through the internal granule layer (Fig. 5e). In addition, only weak staining was observed in the cell bodies of granule cells; intense staining was often recorded between granule cells due to immunoreactivity of axons coursing through the internal granule layer (Fig. 5g). Some deep cerebellar nuclei neurons (Fig. 5f) demonstrated "clusters" of staining reaction associated with the cell surface; this may represent staining of synaptic terminals. Faint staining of Purkinje cell bodies was often observed. Other areas of the brain expressed an intense axonal staining as exemplified by the brain stem, corpus callosum (Fig. 5,i and j), or optic chiasma (not shown). Nuclear, dendritic, or glial staining was not detected with antiserum against brain spectrin(240/235).

The rbc spectrin antibody revealed a totally different distribution of brain spectrin(240/235E). The cerebellum overview (Fig. 6a) exhibited an intense staining of the internal granule layer, a moderate staining of the molecular layer, but little staining of the medullary layer; a few remaining erythrocytes could be recorded in the cerebellum (Fig. 6c). The subcellular localization of staining was confined to the cell bodies and dendrites of neurons, such as the deep cerebellar nuclei neurons (Fig. 6d), Purkinje cells (Fig. 6f), and granule cells (Fig. 6g). Processes extending from the internal granule layer into the molecular layer were observed, and may represent staining of Bergmann glial fibers (Fig. 6e). Neuronal cell bodies and dendrites were prominently stained in other brain regions (Fig. 6, j and k), and cells suggestive of glial cells (Fig. 6, h and i) were observed to be immunoreactive. There was no detectable brain spectrin(240/235E) in axons. Therefore, brain spectrin(240/235) and brain spectrin(240/235E) can be distinguished by their differential staining by rbc and brain spectrin antiserum on Western blots, and distinct localization within neural tissue.

Figure 5. Distribution of the brain spectrin(240/235) in adult mouse brain. Cryostat sections (10 μm) were stained with brain spectrin(240/235) antiserum (after affinity chromatography) followed by peroxidase-conjugated goat anti-rabbit IgG. (a) Low magnification view of the cerebellar cortex. Staining of the medullary layer (MED), the molecular layer (MOL) and internal granule layer (IGL) are demonstrated. PIA, pia. (b) An area similar to that in a stained with preimmune serum. (c) High magnification photomicrograph of the MED demonstrates staining of axons (arrows). (d) Basket fibers (bf) stained in the MOL. (e) Staining of axonal fibers (arrow) coursing through the IGL, with relatively weak staining of granule cells. (f) Staining of a neuronal cell body in the deep cerebellar nuclei, with clusters of stain (arrows) associated with the cell surface. (g) High magnification photomicrograph of granule cells (gc) demonstrates weak cell body staining, with intense staining of
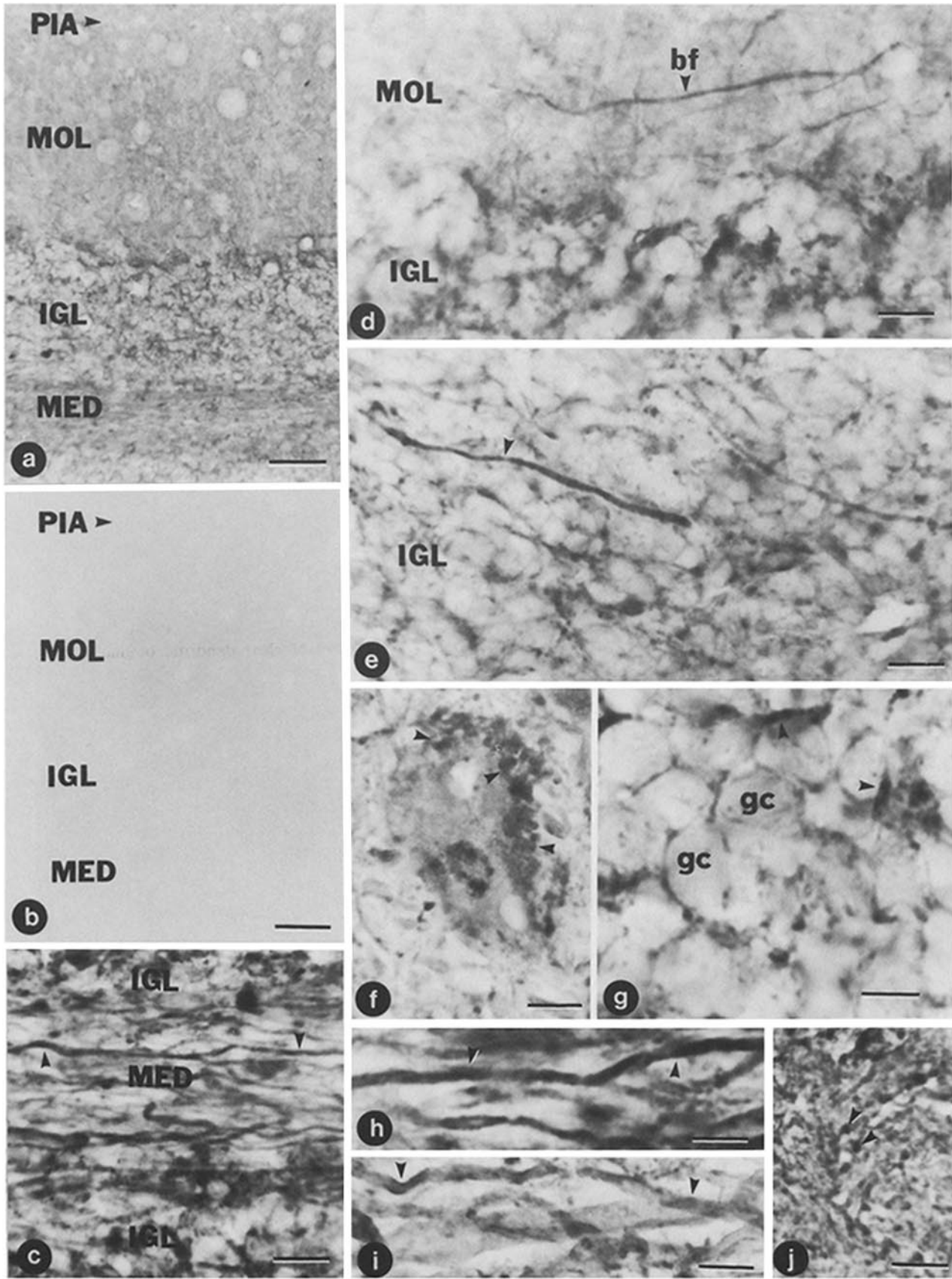
axons cut in cross-section (arrows) which lie between the granule cells. (*h–j*) Axonal (arrows) staining in the cerebellar MED (*h*), brain stem (*i*), and corpus callosum in cross-section (*j*). Antiserum dilution was 1:100 (100 µg IgG/ml). Bars, (*a* and *b*) 50 µm; (*c–f* and *j*) 12.5 µm; and (*g* and *h*) 5 µm.
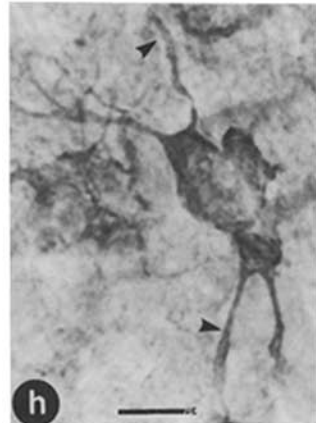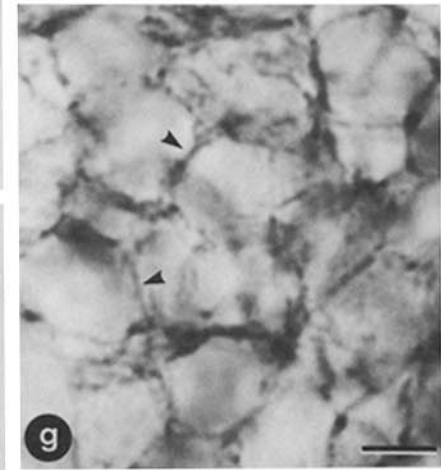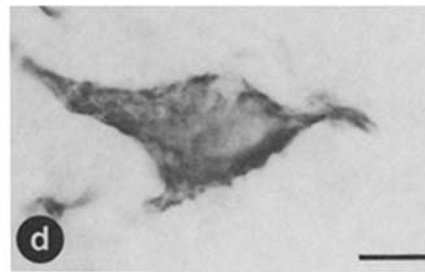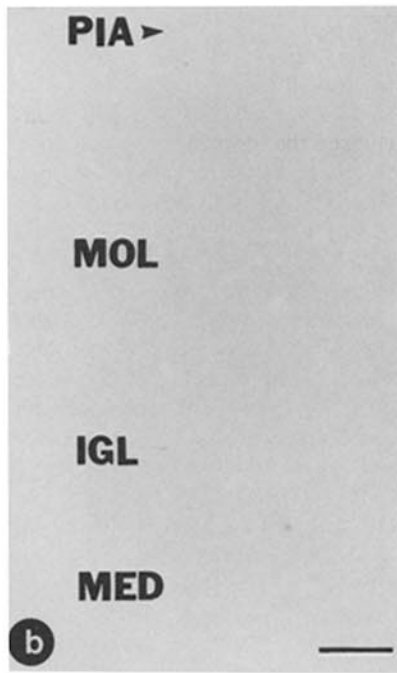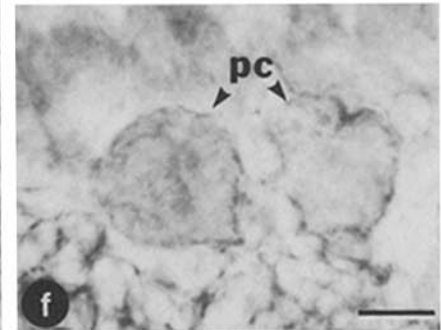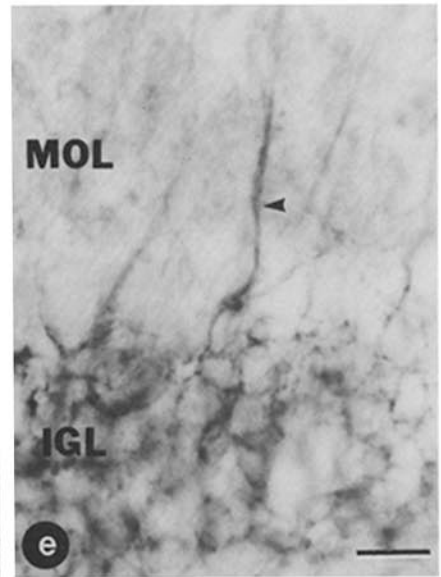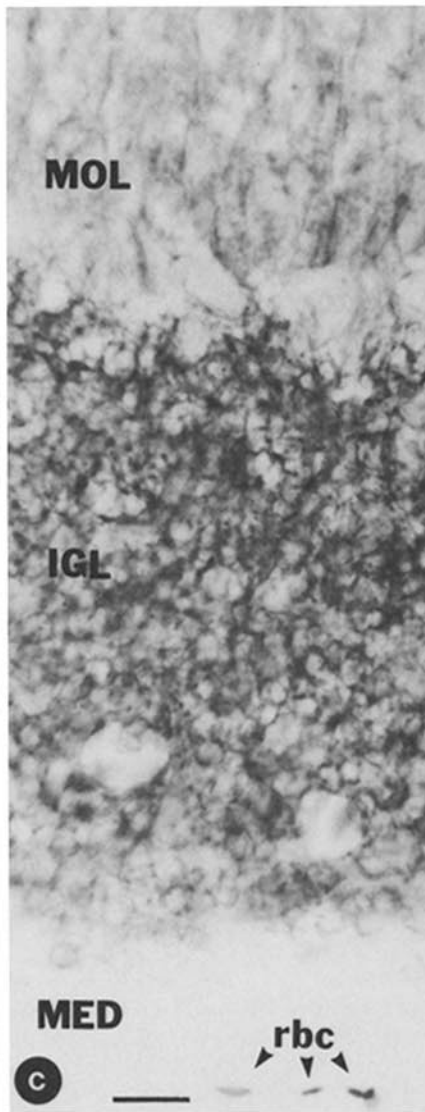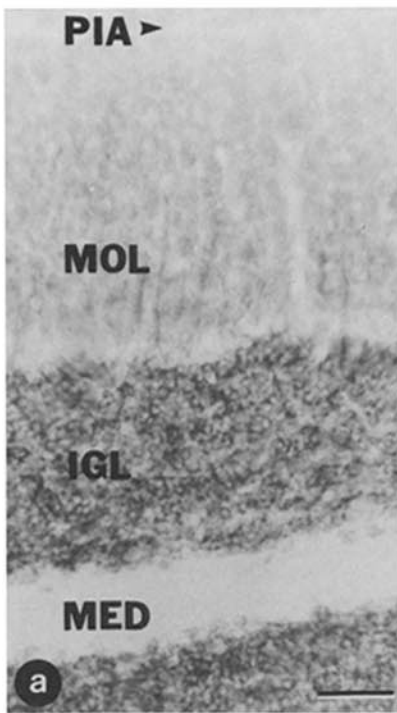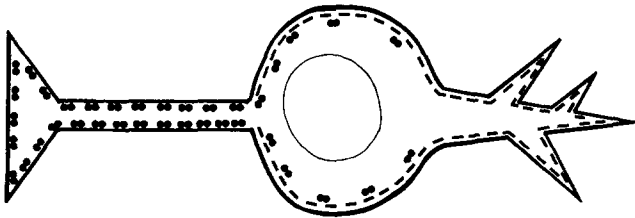
Figure 7. Hypothetical brain spectrin subtype distribution in a neuron. The double dots represent brain spectrin(240/235), most of which is found in axons with less in the cell body. The dashes are the brain spectrin(240/235E) subtype which is localized in the cell body and dendrites.

Discussion

Characterization of Brain Spectrin(240/235) and Brain Spectrin(240/235E)

As summarized in Fig. 7, this report demonstrates that mammalian brain contains at least two spectrin subtypes. (*a*) Brain spectrin(240/235) is enriched in the axons of neurons and to a lesser extent in the cell bodies, but is not found in dendrites. Brain spectrin(240/235) is detected with antiserum directed against spectrin isolated from a crude synaptic/axonal membrane preparation (P2B fraction), but is not detected with antiserum against rbc spectrin. (*b*) Brain spectrin(240/235E) is present in neuronal cell bodies and dendrites, but is excluded from axons. This subtype of brain spectrin is detected specifically by antiserum against mouse rbc spectrin. Unlike the brain spectrin(240/235) subtype, rbc spectrin antiserum also recognizes an immunoreactive analogue of spectrin in certain glial cell types. This analogue may be similar or identical to brain spectrin(240/235E). While we demonstrate here for the first time that mammalian brain contains at least two distinct spectrin subtypes, we want to clearly state that additional subtypes may well be present. We do not as yet know whether these brain spectrin subtypes represent distinct gene products or different posttranslational modifications. Evidence for the presence of multiple spectrin isoforms in mammalian skeletal muscle has recently appeared in abstract form (Shile et al., 1985).

The results presented here suggest a reasonable explanation for the previous conundrum concerning the localization of spectrin within mammalian brain. As brain spectrin(240/235) and brain spectrin(240/235E) are both partially eluted by high and low ionic strength extraction of crude brain homogenates (which has been the standard protocol in this field for brain spectrin isolation), the antibodies which have been previously obtained against mammalian brain spectrin probably recognize both subtypes. When staining the neuronal cell body, dendrites, and axons of guinea pig peripheral nervous tissue (as well as when staining Schwann cells) with antibodies against guinea pig fodrin (brain spectrin), Levine and Willard were most likely detecting both brain spectrin subtypes, whereas Zagon et al. (1984), using the same mouse rbc spectrin antibody used in this report, were specifically detecting the brain spectrin(240/235E) subtype.

Comparison of Mammalian and Avian Brain Spectrin Subtypes

Lazarides and Nelson (1983*a*, *b*) have described two forms of chicken brain spectrin: (*a*) brain spectrin(240/235), which they detected in the cell bodies, axons, and dendrites of neurons at all stages of cerebellar development with an antibody against chicken rbc spectrin 240-kD α subunit; and (*b*) brain spectrin(240/230 or 240/220), a subtype which is antigenically and structurally nearly identical to chicken rbc spectrin, and which is located exclusively on the plasma membrane of the neuronal cell body and dendrites accumulating at the phase of synaptogenesis (Lazarides and Nelson, 1983*a*, *b*). The chicken brain spectrin(240/230 or 240/220) which is detected by Lazarides and Nelson with an antibody against the β subunit of chicken rbc spectrin, appears to have the same location within neurons as does mammalian brain spectrin(240/235E), but contains a 230-kD β' or 220-kD β subunit. The location of chicken brain spectrin(240/235) was in neuronal cell bodies, dendrites, and axons of the cerebellum; this localization differs from mammalian brain spectrin(240/235) which is enriched in axons, present in low quantity in cell bodies, and not present in dendrites. One should keep in mind however that the α subunit of all chicken spectrins is an antigenically and structurally constant subunit (Glenney and Glenney, 1983*a*, *b*; 1984). Therefore, by using an antibody against the α subunit of chicken rbc spectrin, Lazarides and Nelson would have been detecting all chicken brain spectrin subtypes in cerebellum, not only brain spectrin(240/235) as suggested (Lazarides and Nelson, 1983*a*). Despite this caveat, it would appear that there may be differences in the location of spectrin(240/235) subtypes when avian and mammalian brain are compared.

Figure 6. Distribution of brain spectrin(240/235E) in adult mouse brain tissue. Cryostat sections (10 μm) were stained with mouse rbc spectrin antiserum (after affinity chromatography) followed by peroxidase-conjugated goat anti-rabbit IgG. (*a*) Low magnification photomicrograph of the cerebellar cortex. Note the intense staining of the internal granule layer (*IGL*), moderate staining of the molecular layer (*MOL*), and little or no staining of the medullary layer (*MED*). The lack of staining of the MED is striking when compared to Fig. 5*a*. (*b*) Same area as *a* stained with preimmune serum. (*c*) A higher magnification photomicrograph of the cerebellum demonstrates intense staining of individual granule cells within the IGL, and an unstained MED except for the red blood cells (*rbc*) (arrows). (*d*) The cell body and processes of a neuron within the deep cerebellar nuclei are stained. (*e*) The MOL demonstrates only low to moderate staining. Immunoreactive fibers in the MOL (arrows) may be Bergmann glia processes. (*f*) The cytoplasm of Purkinje cells (*pc*) are stained. (*g*) Granule cells show a characteristic cytoplasmic staining pattern between the large cell nucleus and the plasma membrane (arrows). In the cortex of the forebrain, staining of neural cell bodies and processes (arrows) were recorded (*h* and *i*); the cell in *h* was tentatively identified as an oligodendrocyte, while the cell in *i* resembles an astrocyte contacting a blood vessel (*b*). (*j* and *k*) Staining of neuronal cell bodies and dendrites were observed independent of whether frozen cryostat sections (see Materials and Methods) (*j*) or those fixed in 2% paraformaldehyde, 0.25% glutaraldehyde, PBS, pH 9.0 (50-μm vibratome sections) (*k*) of brain were used. Neurons in the region of the vestibular nucleus are shown in both *j* and *k*. Antiserum was diluted 1:100 (100 μg IgG/ml). Bars, (*a* and *b*) 50 μm; (*c*) 20 μm; (*d–f, h–k*) 12.5 μm; and (*g*) 5 μm.

Cytoskeletal Diversity in Neuronal Cellular Compartments

We demonstrate here that brain spectrin(240/235) is primarily an axonal subtype, while brain spectrin(240/235E) is found in dendrites and perikarya. Similarly we have recently demonstrated that there are at least three forms of brain protein 4.1: a cell body/dendrite form (Goodman et al., 1984a), an axonal form, and a unique form in the presynaptic terminals (synapsin I) (for review see Krebs et al., 1986). This compartmental separation of cytoskeletal subtypes or isoforms is not unique to spectrin and related proteins. α- and β-tubulin are present in both mammalian neuronal and glial cell types, but while α- and β-tubulin are both present in neuronal dendrites and glia, β-tubulin is either diminished or modified in parallel fiber axons and in myelinated axons in the white matter (Cumming et al., 1982; 1984). The different α- and β-tubulin isoforms may be of importance in the surface interaction of microtubules with other structural and regulatory cellular components (Sullivan and Wilson, 1984). In addition, the microtubule-associated proteins (MAPs) MAP1, MAP2, and MAP3, all have distinct localization within mammalian neural cells. MAP1 is present in the cell body, in initial axon segments, and throughout the dendritic tree of Purkinje cells; it is less prominent in granule cells, and not present in glial cell types (Huber and Matus, 1984; Bloom et al., 1984, 1985), although the recognition of MAP1 in glial cells seems to depend on the specific monoclonal antibody used. MAP2 is present only in dendrites beyond the initial proximal portion, and is not present in the cell bodies or axons of Purkinje cells or granule cells (Matus et al., 1983; Huber and Matus, 1984). MAP2 was found at fivefold lower concentration relative to tubulin in microtubule preparations of white matter versus gray (Vallee, 1982). MAP3, unlike MAP1 and MAP2, is present in both neuronal and glial cells. In neurons, MAP3 is found exclusively in neurofilament rich axons (myelinated axons, basket fibers) (Huber et al., 1985). The neurofilament proteins of 73 kD and 145 kD are present in axons, cell bodies, and dendrites of mammalian neurons, while the 195-kD neurofilament protein is found primarily in axons (Hirokawa et al., 1984). Actin appears to be concentrated in neuronal dendrites and postsynaptic densities and may be present in different isotypic forms (Matus et al., 1982; Caceres et al., 1983). From the studies cited above it is clear that the dendritic, cell body, and axonal compartments of a neuron contain strikingly different cytoskeletal compositions. It is reasonable to believe that control of the expression of genes coding for these compartment-specific cytoskeletal subtypes (such as brain spectrin[240/235] and brain spectrin[240/235E]), or isoforms of other cytoskeletal proteins, may be at the center of the microdifferentiation process, in which a neuron must produce axonal or dendritic branches, and presynaptic and postsynaptic terminals in regulated sequence and number. Furthermore, the diversity in cytoskeletal proteins is undoubtedly essential to unique functions of neuronal compartments such as translocation of organelles, axonal transport, synaptic transmission, and maintaining the architecture of cell bodies, dendrites, and axons. Brain spectrin(240/235) and brain spectrin(240/235E) may play essential roles in establishing and maintaining these cytoskeletal microdomains within neuronal compartments.

Prospectives for Future Studies

It will be of interest to determine the relative quantities of mammalian brain spectrin(240/235) and brain spectrin(240/235E). This quantitation will be possible only after the purification of both brain spectrin subtypes is complete, for use in quantitative immunodot assays or radioimmunoassays. In addition, before framing appropriate questions concerning the functions of discrete brain spectrin subtypes, we must know the precise intracellular location and contacts of brain spectrin subtypes at the electron microscope level of resolution. These studies which are reported in a separate manuscript (Zagon et al., manuscript submitted for publication), confirm our immunohistochemical observations at the light microscope level. In addition they clearly demonstrate the presence of brain spectrin(240/235) in the presynaptic terminals and brain spectrin(240/235E) in the postsynaptic terminals. Koenig and Repasky (1985) have recently demonstrated the presence of α spectrin at the synaptic junctions of the goldfish Mauthner neuron using an antibody raised against the 240-kD α subunit of chicken rbc spectrin.

An important consequence of this report is that future studies on the functional characteristics of mammalian brain spectrin should consider the necessity of isolating distinct subtypes of brain spectrin. Although we have not yet been able to quantitate the relative amounts of spectrin(240/235) and spectrin(240/235E) in mammalian brain, it is probably no longer sufficient to isolate total mammalian brain spectrin (which contains a co-mixture of the subtypes) and use this material to study binding to syndein/ankyrin, calmodulin, protein 4.1, or actin. We do not know whether distinct brain spectrin subtypes will have different functional characteristics. A more sophisticated approach to brain spectrin function may be necessary in future studies.

References

Bennett, V., J. Davis, and W. E. Fowler. 1982. Brain spectrin, a membrane-associated protein related in structure and function to erythrocyte spectrin. *Nature (Lond.)* 299:126-131.

Bennett, V. 1985. The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54:273-304.

Bloom, G. S., T. A. Schoenfeld, and R. B. Vallee. 1984. Widespread distribution of the major polypeptide component of MAP1 (microtubule-associated protein 1) in the nervous system. *J. Cell Biol.* 98:320-330.

Bloom, G. S., F. C. Luca, and R. B. Vallee. 1985. Microtubule-associated protein 1B: Identification of a major component of the neuronal cytoskeleton. *Proc. Natl. Acad. Sci. USA.* 82:5404-5408.

Burridge, K., T. Kelly, and P. Mangeat. 1982. Nonerythrocyte spectrins: actin-membrane attachment proteins occuring in many cell types. *J. Cell Biol.* 95:478-486.

Caceres, A., M. R. Payne, L. I. Binder, and O. Steward. 1983. Immunocytochemical localization of actin and microtubule-associated protein MAP2 in dendritic spines. *Proc. Natl. Acad. Sci. USA.* 80:1738-1742.

Cheney, R., N. Hirokawa, J. Levine, and M. Willard. 1983. Intracellular movement of fodrin. *Cell Motility.* 3:649-655.

Cumming, R., R. D. Burgoyne, and N. A. Lytton. 1982. Differential immunocytochemical localisation of α-tubulin and β-tubulin in cerebellum using monoclonal antibodies. *Cell Biol. Int. Rep.* 6:1047-1053.

Cumming, R., R. D. Burgoyne, and N. A. Lytton. 1984. Immunocytochem-

ical demonstration of α-tubulin modification during axonal maturation in the cerebellar cortex. *J. Cell Biol.* 98:347–354.

Glenney, J. R., and J. P. Glenney. 1983a. Fodrin is the general spectrin-like protein found in most cells whereas spectrin and TW protein have a restricted distribution. *Cell.* 34:503–512.

Glenney, J. R., and J. P. Glenney. 1983b. Spectrin, fodrin and TW260/240: a family of related proteins lining the plasma membrane. *Cell Motil.* 3:671–682.

Glenney, J. R., and J. P. Glenney. 1984. Comparison of spectrin isolated from erythrocyte and non-erythroid sources. *Eur. J. Biochem.* 144:529–539.

Glenney, J. R., J. P. Glenney, and K. Weber. 1982. Erythroid spectrin, brain fodrin, and intestinal brush border proteins (TW260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit. *Proc. Natl. Acad. Sci. USA.* 79:4002–4005.

Goodman, S. R., L. A. Casoria, D. B. Coleman, and I. S. Zagon. 1984a. Identification and location of brain protein 4.1. *Science (Wash. DC).* 224:1433–1436.

Goodman, S. R., S. A. Weidner, M. E. Eyster, and J. J. Kesselring. 1982a. Binding of spectrin to a hereditary spherocyte membranes. *J. Mol. Cell. Cardiol.* 14:91–97.

Goodman, S. R., J. Yu, C. F. Whitfield, E. N. Culp, and E. J. Posnak. 1982b. Erythrocyte membrane skeletal protein bands 4.1a and b are sequence-related phosphoproteins. *J. Biol. Chem.* 257:4564–4569.

Goodman, S. R., and I. S. Zagon. 1984. Brain spectrin: a review. *Brain Res. Bull.* 13:813–832.

Goodman, S. R., and I. S. Zagon. 1986. The neural cell spectrin skeleton: a review. *Am. J. Physiol.* 250:C347–C360.

Goodman, S. R., I. S. Zagon, and R. R. Kulikowski. 1981. Identification of a spectrin-like protein in nonerythroid cells. *Proc. Natl. Acad. Sci. USA.* 78:7570–7574.

Goodman, S. R., I. S. Zagon, C. F. Whitfield, L. A. Casoria, P. J. McLaughlin, and T. L. Laskiewicz. 1983. A spectrin-like protein from mouse brain membranes: immunological and structural correlations with erythrocyte spectrin. *Cell Motil.* 3:635–647.

Goodman S. R., I. S. Zagon, C. F. Whitfield, L. A. Casoria, S. B. Shohet, S. E. Bernstein, P. J. McLaughlin, and T. L. Laskiewicz. 1984b. A spectrin-like protein from mouse brain membranes: phosphorylation of the 235,000 dalton subunit. *Am. J. Physiol.* 16:C61–C73.

Gray, E. G., and V. P. Whittaker. 1962. The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived from homogenization and centrifugation. *J. Anat.* 96:79–88.

Hawkes, R., E. Niday, and A. Matus. 1982. Monoclonal antibodies identify neural antigens. *Proc. Natl. Acad. Sci. USA.* 79:2410–2414.

Hirokawa, N., M. A. Glicksman, and M. B. Willard. 1984. Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. *J.*

Cell Biol. 98:1523–1536.

Huber, G., D. Alaimo-Beuret, and A. Matus. 1985. MAP3: Characterization of a novel microtubule-associated protein. *J. Cell Biol.* 100:496–507.

Huber, G., and A. Matus. 1984. Differences in the cellular distribution of two microtubule-associated proteins, MAP1 and MAP2, in rat brain. *J. Neurosci.* 4:151–160.

Jahn, R., W. Schiebler, and P. Greengard. 1984. A quantitative dot-immunobinding assay for proteins using nitrocellulose membrane filters. *Proc. Natl. Acad. Sci. USA.* 81:1684–1687.

Kay, M. M. B., S. R. Goodman, K. Sorensen, C. F. Whitfield, P. Wong, L. Zaki, and V. Rudloff. 1983. Senescent cell antigen is immunologically related to band 3. *Proc. Natl. Acad. Sci. USA.* 80:1631–1635.

Koenig, E., and E. Repasky. 1985. A regional analysis of α-spectrin in the isolated Mauthner neuron and in isolated axons of the goldfish and rabbit. *J. Neurosci.* 5:705–714.

Krebs, K. E., I. S. Zagon, and S. R. Goodman. 1986. Brain protein 4.1 and synapsin I are related neural proteins. *News in Physiological Sciences.* In press.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 226:680–685.

Lazarides, E., and W. J. Nelson. 1983a. Erythrocyte and brain forms of spectrin in cerebellum: distinct membrane-cytoskeletal domains in neurons. *Science (Wash. DC).* 220:1295–1296.

Lazarides, E., and W. J. Nelson. 1983b. Erythrocyte form of spectrin in cerebellum: appearance at a specific stage in the terminal differentiation of neurons. *Science (Wash. DC).* 222:931–933.

Levine, J., and M. Willard. 1983. Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* 90:631–643.

Matus, A., M. Ackermann, G. Pehling, H. R. Byers, and K. Fujiwara. 1982. High actin concentrations in brain dendritic spines and postsynaptic densities. *Proc. Natl. Acad. Sci. USA.* 79:7590–7594.

Matus, A., G. Huber, and R. Bernhardt. 1983. Neuronal microdifferentiation. *Cold Spring Harbor Symp. Quant. Biol.* 47:775–782.

Repasky, E. A., B. L. Granger, and E. Lazarides. 1982. Widespread occurrence of avian spectrin in nonerythroid cells. *Cell.* 29:821–833.

Shile, P., A. Harris, and J. Morrow. 1985. Human skeletal muscle contains unique tissue specific spectrin isoforms. *Fed. Proc.* 44:742a. (Abstr.)

Sullivan, K. F., and L. Wilson. 1984. Developmental and biochemical analysis of chick brain tubulin heterogeneity. *J. Neurochem.* 42:1363–1371.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Vallee, R. B. 1982. Taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). *J. Cell Biol.* 92:435–442.

Zagon, I. S., P. J. McLaughlin, and S. R. Goodman. 1984. Localization of spectrin in mammalian brain. *J. Neurosci.* 4:3089–3100.