

A New 440-kD Isoform Is the Major Ankyrin in Neonatal Rat Brain

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Abstract. This report describes initial characterization of a 440-kD isoform of brain ankyrin (ankyrin_B) representing an alternatively spliced mRNA product of the gene encoding the major isoform of ankyrin in adult human brain (Otto, E., M. Kunimoto, T. McLaughlin, V. Bennett, *J. Cell Biology*, 114:241–253). Northern and immunoblot analyses indicate that 440-kD ankyrin_B includes the spectrin and membrane-binding domains as well as a regulatory domain of the major 220-kD isoform. 440-kD ankyrin_B contains, in addition, a sequence of a predicted size of 220 kD which is inserted between the regulatory domain and spectrin/membrane-binding domains. 440-kD ankyrin_B has properties expected of a peripherally associated membrane-skeletal protein: it is exclusively present in the particulate fraction of brain homogenates, is extracted with NaOH, and remains associated with

Triton-X-100-resistant structures. Expression of 440-kD ankyrin_B in rat brain began at birth before other ankyrins could be detected, peaked 10 d after birth, and then decreased progressively to 30% of the maximum in adults. Expression of the 220-kD ankyrin_B and ankyrin_R (erythroid ankyrin) began ~10 d after the 440-kD isoform, increased rapidly between 10 and 15 d after birth, and finally achieved their maximal levels in adults. 440-kD ankyrin_B is present in approximately equivalent amounts in all regions of neonatal brain while in adult brain it is present in highest levels in cerebellum and lowest in brain stem. 440-kD ankyrin_B was localized by immunofluorescence in regions of neonatal and adult brain containing primarily dendrites and unmyelinated axons. 440-kD ankyrin_B thus may play a specialized role in neuronal processes.

ANKYRINS constitute a family of intracellular structural proteins that are candidates to couple a variety of integral membrane proteins to the spectrin skeleton (reviewed in references 4 and 6). Integral proteins that are currently known to associate with ankyrins in *in vitro* assays and are co-localized with ankyrin in tissues include the anion exchanger (5, 12), the Na/K ATPase (15, 21, 23), and the voltage-dependent Na channel (27, 13, 17). The detailed protein interactions of ankyrins are best understood in human erythrocytes where this protein forms a ternary complex involving the cytoplasmic domain of anion exchanger and the beta subunit of spectrin. Erythrocyte ankyrin contains three domains that participate in this complex: a 89-kD membrane-binding domain that contains the binding site for the anion exchanger (9), a 62-kD domain that associates with spectrin (2), and a regulatory domain of 55 kD (14).

Ankyrins are present in a number of tissues and are expressed at especially high levels in vertebrate brain, where this family has been estimated to comprise from 0.5–1 per-

cent of the total membrane protein (3, 8). A major isoform of ankyrin in brain tissue has been purified that shares functional activities and a similar domain structure with erythrocyte ankyrin (8). Brain tissue also contains an isoform of ankyrin encoded by the same gene (*ANK1*) as erythrocyte ankyrin (referred to in this report as ankyrin_R), which is missing in brains of mutant mice with defective expression of this gene (16, 26). A distinct isoform of ankyrin is localized at the nodes of Ranvier in myelinated nerves; this isoform persists in the ankyrin_R-deficient mice and thus is likely to be encoded by a gene distinct from *ANK1* (16). In addition to multiple genes, alternative splicing of pre-mRNAs have also been shown to provide additional ankyrin variants (18, 20, 24). The diversity of ankyrins is consistent with their proposed role as adaptors between multiple membrane proteins and the spectrin skeleton in a number of different cells.

The amino acid sequence of the major 220-kD ankyrin of adult human brain (a product of the *ANK2* gene) has recently been deduced from analysis of cDNAs (24). This information together with the primary sequence of ankyrin_R (18, 20) defines consensus features of the ankyrin family. These proteins both contain highly conserved regions that include a series of 22 consecutive repeats of 33 residues in the

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membrane-binding domains, and the COOH-terminal portion of the spectrin-binding domains. The regulatory domains of the two ankyrins are quite different, consistent with the observations of distinct membrane attachment sites for these proteins (10).

Additional cDNAs representing an alternatively spliced mRNA product of the *ANK2* gene were characterized during the isolation of cDNAs encoding the 220-kD ankyrin_B. These alternative forms shared NH₂-terminal sequences with the 220-kD ankyrin_B, but also included unique sequence unrelated to either this form of brain ankyrin or to ankyrin_R (24). cDNA encoding the unique portion of the new form of ankyrin_B has only been partially cloned and sequenced. However, a 440-kD polypeptide that cross-reacted with antibody against the conserved portion of ankyrin_B was identified as a candidate for the protein product of the new ankyrin sequence (24). A 13-kb mRNA hybridized with probes against the new sequence as well as all of the established ankyrin domains, suggesting the possibility that the 13-kb mRNA represents an alternatively spliced message that encodes a new ankyrin and includes the sequence of 220-kD ankyrin_B of adult brain (24). In this paper, we present evidence that the 13-kb mRNA encodes a polypeptide of 440 kD which contains the sequence of 220-kD ankyrin_B plus additional protein sequence representing ~220 kD that is unique to the 440-kD ankyrin_B. The 440-kD isoform of ankyrin_B (referred to as 440-kD ankyrin_B) is the major form of ankyrin detected in developing rat brain and is highly expressed in the molecular layers of the cerebellum of neonatal as well as of adult rats. Since the molecular layer is enriched in unmyelinated axons and dendrites of neurons, 440-kD ankyrin_B may play a specialized role in these structures.

Materials and Methods

Materials

Carrier-free Na¹²⁵I, [α -³²P]dCTP and Multiprime DNA-labeling system were purchased from Amersham Corp. (Arlington Heights, IL). Diisopropyl fluorophosphate, leupeptin, pepstatin A, DTT, PMSF, EDTA, and EGTA were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose paper and electrophoresis reagents were from Bio-Rad (Richmond, CA). Protein A, protein A-Sepharose 6MB, CNBr-activated Sepharose CL-4B, Oligo (dT) cellulose, isopropyl β -D-thiogalactoside, and a Mono Q column were from Pharmacia LKB Biotechnology (Piscataway, NJ). EcoRI, SalI, and XhoI were from International Biotechnologies Inc. (New Haven, CT).

Recombinant Proteins

cDNA sequences of regions 1 (nucleotides 4,330–5,013) and 2 (nucleotides 4,306–6,163) of brain ankyrin (see Fig. 1) were amplified by the polymerase chain reaction using appropriate primers containing engineered restriction sites for EcoRI (5' end of region 1 fragment and 5' and 3' ends of the region 2 fragment) and SalI (3' end of the region 1 fragment). Amplified DNAs were digested with the restriction enzymes, purified by preparative agarose gel electrophoresis followed by Gene Clean (Bio 101, La Jolla, CA), and subcloned into the multicloning site of pGEMEX (Promega Corp., Madison, WI) containing the T7 promoter (30). Plasmids were originally amplified within JM109 cells, and then introduced into BL21 DE(3) pLys or JM109 DE(3) cells, which are expressor strains containing a chromosomal gene encoding the viral T7 RNA polymerase under control of the lacZ promoter. Protein expression was induced by the addition of isopropyl β -D-thiogalactoside at a final concentration of 0.5 mM. Expressed proteins were exclusively recovered in inclusion bodies of the bacteria, which were isolated as described (11) and solubilized in a minimum volume of 8 M urea containing 1 mM NaEDTA, 10 mM glycine, 1 mM NaN₃ and 10 mM sodium phosphate, pH 7.4. Recombinant proteins, which are fusion proteins

containing the viral gene 10 protein at their NH₂ termini, were purified by chromatography using a Mono Q column in the presence of 6 M urea. Proteins were further purified with preparative SDS-polyacrylamide gel electrophoresis and electroelution. The gene 10 product was prepared in the same way using pGEMEX without inserts.

Antibodies

Antisera against fusion proteins derived from regions 1 and 2 (see Fig. 1) were raised in rabbits. Affinity-purified antibodies specific for polypeptides comprising region 1 and 2 were prepared by affinity chromatography using Sepharose CL-4B coupled with either of these fusion proteins after passing sera through a column of viral gene 10 product coupled Sepharose CL-4B to remove reactivity against the gene 10 portion of the fusion proteins. Control Ig was prepared with a protein A-Sepharose 6MB column from the sera recovered as the breakthrough fraction of a region 2-affinity column. Affinity-purified rabbit antibodies were prepared against ankyrin_R of human erythrocytes that either reacted with other ankyrins (8), or were specific for ankyrin_R (16), and against brain spectrin (7). mAbs against synaptophysin and myelin basic protein were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Polyclonal rabbit anti-mouse Ig was obtained from Sigma Chemical Co. Rhodamine-labeled goat anti-rabbit Ig was from Cappel Laboratories (Cochranville, PA).

Gel Electrophoresis and Immunoblot Analysis

Brain tissue was removed from Sprague-Dawley rats of various ages indicated in the text and homogenized with a Polytron (Brinkmann Instruments Inc., Westbury, NY) in 9 vol of homogenization buffer (0.32 M sucrose, 2 mM NaEGTA, 1 mM NaN₃, 5 μ g/ml pepstatin A, 5 μ g/ml leupeptin, 200 μ g/ml PMSF, 0.01% [vol/vol] diisopropyl fluorophosphate, pH 7.5). Homogenates were mixed with an equal volume of SDS-sample buffer (7) directly or after differential centrifugation and heated at 65°C for 20 min. Samples were electrophoresed on SDS-polyacrylamide gradient gels and immunoblotted using ¹²⁵I-labeled protein A to visualize antibodies as described (7). In the case of mAbs (mouse Ig G₁), rabbit anti-mouse IgG was used as a second antibody.

RNA Preparation and Northern Blot Analysis

Total RNA was extracted from brains of adult and 0-, 10- and 26-d-old rats using RNazol methods (Cinna/Biotech, Friendswood, TX) and poly (A)⁺ RNA was isolated with oligo (dT) cellulose column. Poly (A)⁺ RNAs were fractionated by formaldehyde/1.2% agarose gel electrophoresis and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH) as described (31). A RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used to calculate the relative size of ankyrin messages. Membranes were hybridized with radiolabeled brain ankyrin cDNA inserts corresponding to regions 1 (nucleotides 4,344–5,028), 2 (nucleotides 4,351–4,930), and common probes (nucleotides 583–2,192) and (nucleotides 2,841–4,763) (see reference 24 for actual sequence) prepared with [³²P]dCTP using Multiprime DNA-labeling system and washed under two different stringent conditions (0.1 \times SSC at 65°C for the region 2 and common probes and 1 \times SSC at 45°C for the region 1 probe) before autoradiography.

Immunocytochemical Procedures

Adult and 10-d-old rats were perfused with 1% NaNO₃, pH 7.5, containing heparin (50 U/ml), and then with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.5. Whole brains were removed, separated into forebrain, cerebellum and brain stem, and placed in fixative overnight. The samples were cryoprotected with 25% sucrose, frozen in liquid nitrogen-cooled isopentane, and cut at -20°C into 10- μ m-thick sections. Cryosections were mounted on ovalbumin-coated glass slides, rinsed in PBS and immunostained using antibodies at 4 μ g/ml in the presence of 0.1% Triton X-100 by indirect immunofluorescence as described previously (17).

Results

440-kD Ankyrin_B Is Encoded by a 13-kb mRNA That Includes 220-kD Ankyrin_B Sequence

The mRNAs corresponding to regions 1 and 2 to ankyrin_B cDNA sequences were identified by Northern blots of rat

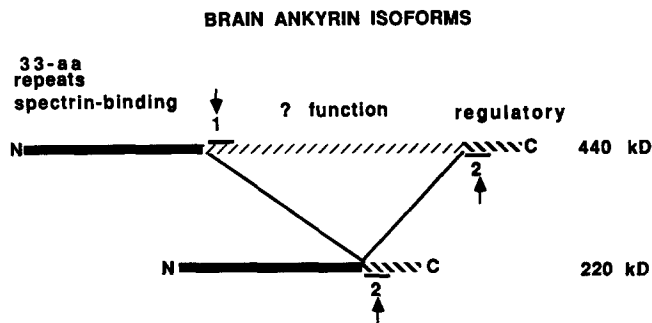


Figure 1. Model for domain organization of 440- and 220-kD brain ankyrin isoforms (referred to in the text as ankyrin_B). Arrows mark the location of regions of cDNA used for preparation of probes for Northern blots and to express proteins for preparation of antibodies.

brain tissue with probes against various portions of human ankyrin_B cDNA (see Fig. 2; and Fig. 1 for location of probes). Probe 1 hybridized to only a large mRNA of ~13 kb, a size identical to that detected with probes for the 33-residue repeat domain, the spectrin-binding domain, and probe 2 sequences derived from the 3' regulatory domain of ankyrin_B (24; and Fig. 2). These results strongly suggest that a single 13-kb mRNA includes all of the functional domains of established ankyrins and, in addition, the unique sequence represented by probe 1. Probes specific for the regulatory domain of 220-kD ankyrin_B (probe 2) and the spectrin domain also hybridized with mRNAs of 9 kb as well as smaller mRNAs of 6 kb and a minor form of 4 kb (Fig. 1; 24). Because the two smallest mRNAs did not hybridize with probes against the 33-residue repeat domain, they do not encode the 220-kD ankyrin and thus most likely repre-

sent additional alternatively spliced variants of ankyrin_B. Thus, the 220-kD form of ankyrin_B is encoded by either a 9- or 13-kb transcript.

The polypeptides corresponding to ankyrin_B mRNAs were detected using antibodies prepared against recombinant polypeptides encoded by cDNAs corresponding to regions 1 and 2 (24) (Fig. 3, and Fig. 1 for location of cDNAs). Polyclonal antibodies specific for polypeptides derived from regions 1 and 2 were affinity purified from antisera obtained from rabbits immunized with the corresponding fusion proteins (see Materials and Methods). These antibodies are highly specific: antibodies against region 1 and region 2 react only with their corresponding polypeptides (Fig. 3). Moreover, neither antibody cross-reacted with ankyrin_R of human erythrocytes or with the viral gene 10 product, which was a component of the recombinant polypeptides used as antigens (Fig. 3).

Antibody against the region 1 recombinant polypeptide recognized one major polypeptide of 440 kD in rat brain (Fig. 4 *b*). The large size of this polypeptide was initially a surprise based on other examples of ankyrins, although it is consistent with the 13-kb mRNA that hybridized with the region 1 DNA probe (Fig. 2). The 440-kD polypeptide contains other portions of ankyrin sequence since it also cross-reacted with antibody against the NH₂ terminal domain of 220-kD ankyrin_B of adult brain (24), and with a general Ig that recognizes ankyrins in multiple tissues (Fig. 4, *h* and *i*). Analysis of mRNAs indicates that the 13-kb message encoding 440-kD ankyrin_B includes the 3' portion of the sequence of the 220-kD adult form of ankyrin_B, and implies that the 440-kD polypeptide should also cross-react with antibody against the region 2 recombinant polypeptide. As expected, antibody against region 2 recognized the 440-kD polypeptide in addition to the major 220-kD ankyrin_B polypeptide.

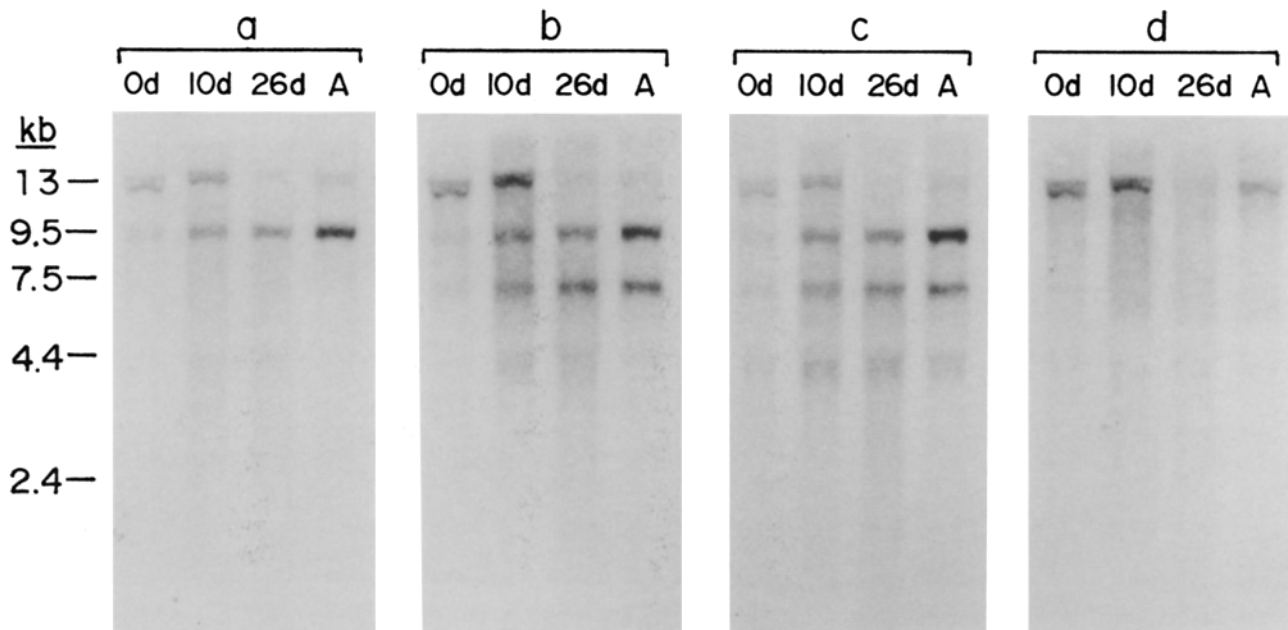


Figure 2. Northern blot analysis of rat brain poly(A)⁺ RNA with probes derived from various regions of ankyrin_B cDNA. Poly(A)⁺ RNAs prepared from brains of 0-, 10- and 26-d-old rats (lanes *0d*, *10d*, and *26d*) and adult (lane *A*) rats were electrophoresed on formaldehyde/agarose gel and transferred to nitrocellulose filter paper. Blots were hybridized with ³²P-labeled cDNA probes derived from the common membrane or spectrin-binding regions (*a* or *b*, respectively), region 2 regulatory domain sequence (*c*), or region 1 sequence (*d*) (see Fig. 1 for location of probes). Nucleotide boundaries of the cDNAs used as probes are described in Materials and Methods.

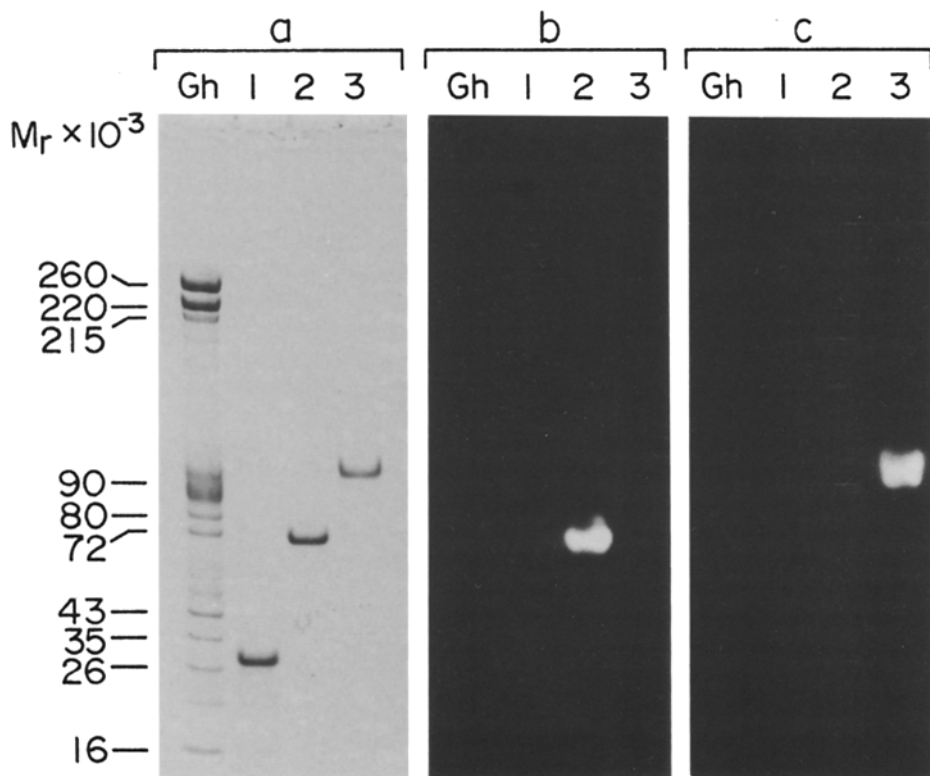


Figure 3. Specificity of affinity-purified antibodies against recombinant polypeptides derived from regions 1 and 2 of ankyrin_B. Purified T7 gene 10 protein (lane 1), region 1 and region 2 recombinant proteins (see Fig. 1 for location of cDNAs) (lanes 2 and 3, respectively) were electrophoretically transferred to nitrocellulose filter after electrophoresis on SDS-polyacrylamide gels. Human erythrocyte ghost proteins (*Gh*) were processed in the same way. Immunoreactivity of these proteins against affinity-purified Ig against region 1 (*b*) and region 2 (*c*) polypeptides were examined by immunoblotting as described in Materials and Methods. A Coomassie blue stained gel pattern is also shown (*a*).

Antibody against region 2 also reacted with a minor polypeptide of 150 kD (Fig. 4 *c*).

It is conceivable that the 440-kD brain polypeptide recognized by antibodies against regions 1 and 2 recombinant

polypeptides actually represents two distinct polypeptides that comigrate on SDS gels. Such a possibility was examined by immunoprecipitating the 440-kD polypeptide with antibody against region 1 recombinant polypeptide followed by

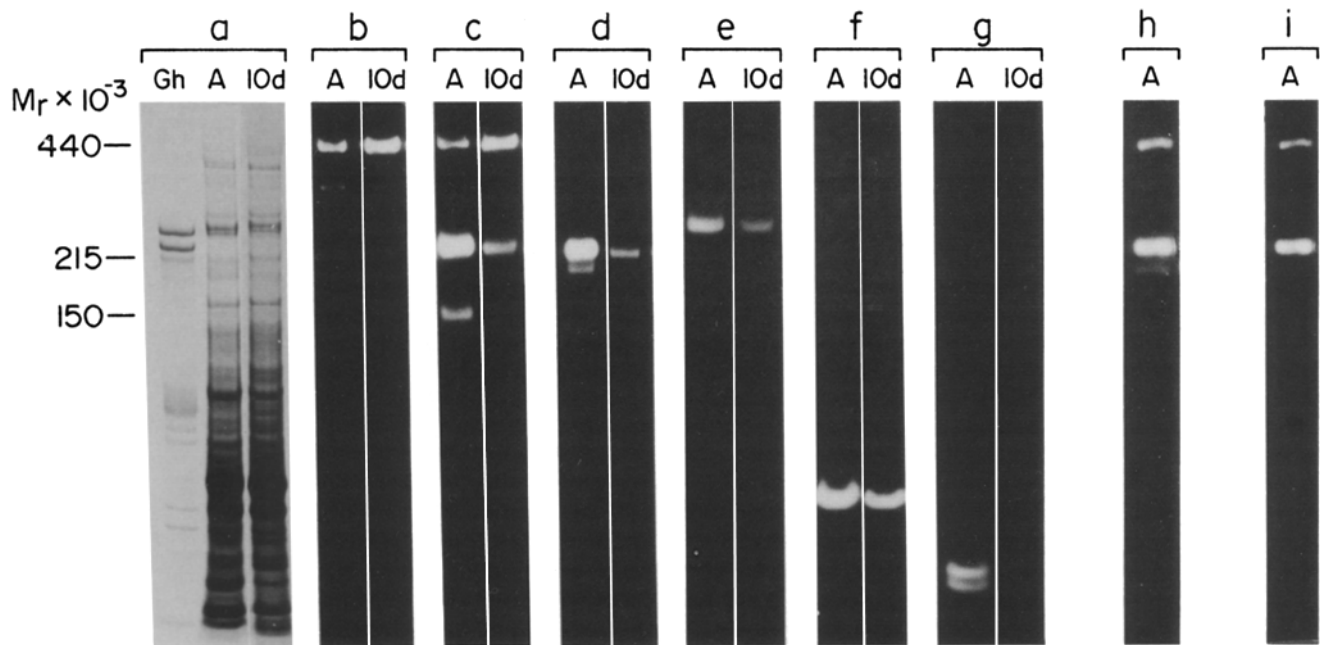


Figure 4. Immunoreactive ankyrin isoforms in adult and 10-d-old rat brains. Total homogenate proteins of adult (*A*) and 10-d-old (*10d*) rat brains were separated with SDS-PAGE and either stained for proteins with Coomassie blue (*a*) or electrophoretically transferred to nitrocellulose filter paper. Immunoreactive ankyrin isoforms were detected using antibodies against region 1 (*b*), or region 2 (*c*) of ankyrin_B, ankyrin_R (*d*), human erythrocyte ankyrin (general reactivity with ankyrins) (*h*), and the common NH₂-terminal region of ankyrin_B (24) (*i*). Immunoreactive forms of brain spectrin (*e*), synaptophysin (*f*), and myelin basic protein (*g*) were also detected as described in Materials and Methods. (*Gh*, human erythrocyte ghost).

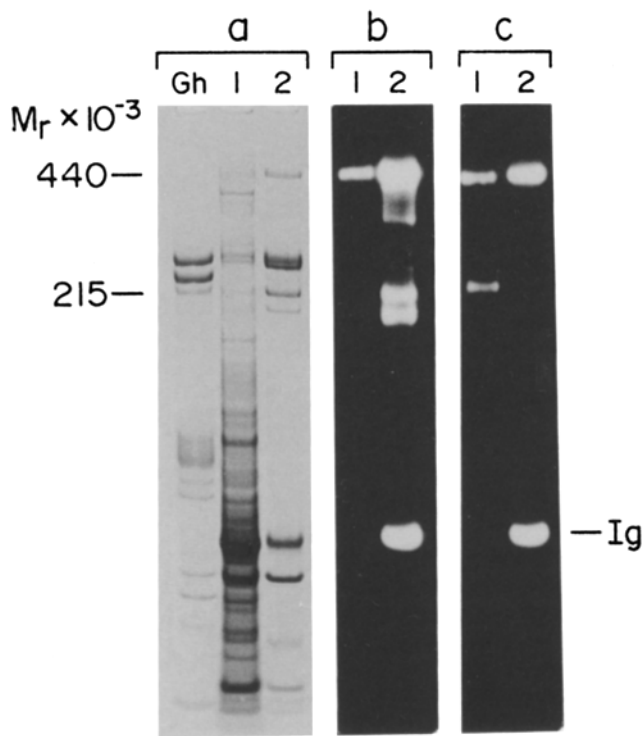


Figure 5. 440- and 220-kD forms of ankyrin_B share protein epitopes. Postnuclear membrane fractions (30,000 g pellets) were isolated from brains of 26-d-old rats and solubilized with 0.1% SDS in buffer 1 (0.15 M NaCl, 1 mM NaEDTA, 0.2% Triton X-100, 1 mM NaN₃, and 10 mM NaPO₄, pH 7.4) in the presence of 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 200 μg/ml PMSF, and 0.01% [vol/vol] diisopropyl fluorophosphate at room temperature. After adding Triton X-100 at final concentration of 1% (wt/vol), insoluble material was removed by centrifugation at 10,000 g for 10 min at 4°C. The supernatant was incubated with antibody against 440-kD ankyrin_B (2 μg/ml) for 5 h and then with protein A-Sepharose 6MB for additional 15 h at 4°C. The Sepharose beads were washed once with buffer 1 and once with 2 M urea, 0.1 M glycine, 1% Triton X-100, and 1 mM NaN₃. Immunoprecipitated proteins and antibodies were eluted into SDS-sample buffer by heating at 65°C for 20 min. Postnuclear brain membranes (lanes 1) and immunoprecipitated proteins (lanes 2) were separated on SDS-PAGE, and either stained with Coomassie blue (a) or transferred to nitrocellulose filter paper and immunoblotted using antibodies against either 440-kD ankyrin_B (b) or 440/220/150-kD ankyrin_B polypeptides (c).

SDS-PAGE and immunoblotting with antibody against the region 2 recombinant polypeptide (Fig. 5). The immunoprecipitated 440-kD polypeptide cross-reacted strongly with antibody against region 2 (Fig. 5), demonstrating that the same 440-kD polypeptide is recognized by both antibodies. Antibody against region 1 recombinant polypeptide also cross-reacted with two major protein bands of 220 kD in addition to the 440-kD protein. The 220-kD polypeptides were not recognized by region 2 antibody, suggesting two possibilities: they may be degradation products of the 440-kD polypeptide generated during the immunoprecipitation process, or they may represent additional products of alternative splicing of mRNA that were highly concentrated during immunoprecipitation. Interestingly, spectrin was consistently co-precipitated by antibody against the 440-kD ankyrin, as

would be expected since the 440-kD isoform is predicted to contain a spectrin-binding domain.

A working model for the organization of 440-kD ankyrin_B primary amino acid sequence based on Northern blots and analysis with antibodies (Fig. 1) places the 440-kD isoform-specific sequence between the NH₂-terminal and COOH-terminal regions, which are conserved between 440- and 220-kD forms of brain ankyrin. Such an arrangement would most likely result from alternative splicing of pre-mRNAs. This model is consistent with recently described cDNA clones that overlap between 440-kD brain ankyrin-specific sequence and the conserved 5' region (24). Clones that extend across the 440-kD-specific region to the 3' portion of 220-kD brain ankyrin have been isolated (W. Chan and V. Bennett, unpublished data). The length of the 440-kD ankyrin_B-specific sequence of 220 kD as estimated from the apparent size of the polypeptide on SDS gels is consistent with the length of the recently isolated 440-specific cDNA of 6.2 kb (W. Chan and V. Bennett, unpublished data). The predicted inserted sequence required to generate the 440-kD form of ankyrin represents an unusual example of mRNA splicing where the additional sequence is nearly as large as the alternative protein. Antibodies against recombinant brain polypeptides will be referred to in the remainder of this paper in terms of their reactivity with brain polypeptides: antibodies against region 1 react with the 440-kD ankyrin_B while antibodies against region 2 react with 440/220/150-kD ankyrin_B polypeptides.

Expression of Ankyrin Isoforms in Developing Rat Brain

Comparison of ankyrin_B mRNAs in rats of different ages revealed that expression of 13 and 9/6 kb mRNAs are developmentally regulated according to different programs. The 13-kb mRNA, which encodes the 440-kD isoform, appeared first, and was detectable as early as at birth (Fig. 2). The 13-kb mRNA subsequently peaked around day 10 and subsided to <20% of maximal levels in adults. The fall in 13-kb mRNA occurred abruptly with the greatest change between day 10 and day 26 (Fig. 2). The 9- and 6-kb mRNAs were present in low amounts at birth and subsequently increased until maximal levels were achieved in adults (Fig. 2).

Relative levels expression of 440- and 220-kD ankyrin polypeptides was examined during neonatal development with the antibodies described above (Fig. 3–5). Immunoblots were performed using radiolabeled protein A to detect Ig, and the amount of label estimated by densitometry of autoradiograms. Care was taken to use autoradiograms that were not overexposed. Data were normalized with respect to amount of protein as determined by scanning a Coomassie blue-stained gel, and are expressed as per cent of the maximal value for each protein. The 440-kD ankyrin_B polypeptide exhibited the same biphasic behavior observed for the 13-kb mRNA: low expression at birth that peaked around days 10–13 and subsequently fell to about 30% of maximal levels in adults. In contrast, the 220-kD ankyrin_B polypeptide was present in very low amounts at birth and increased continuously until adulthood. The period of most rapid accumulation of the 220-kD polypeptide occurred between days 10 and 15, or ~10 d later than expression of the 440-kD polypeptide (Fig. 6). Because this pattern parallels the accumulation of the 9-kb transcript, it is reasonable to con-

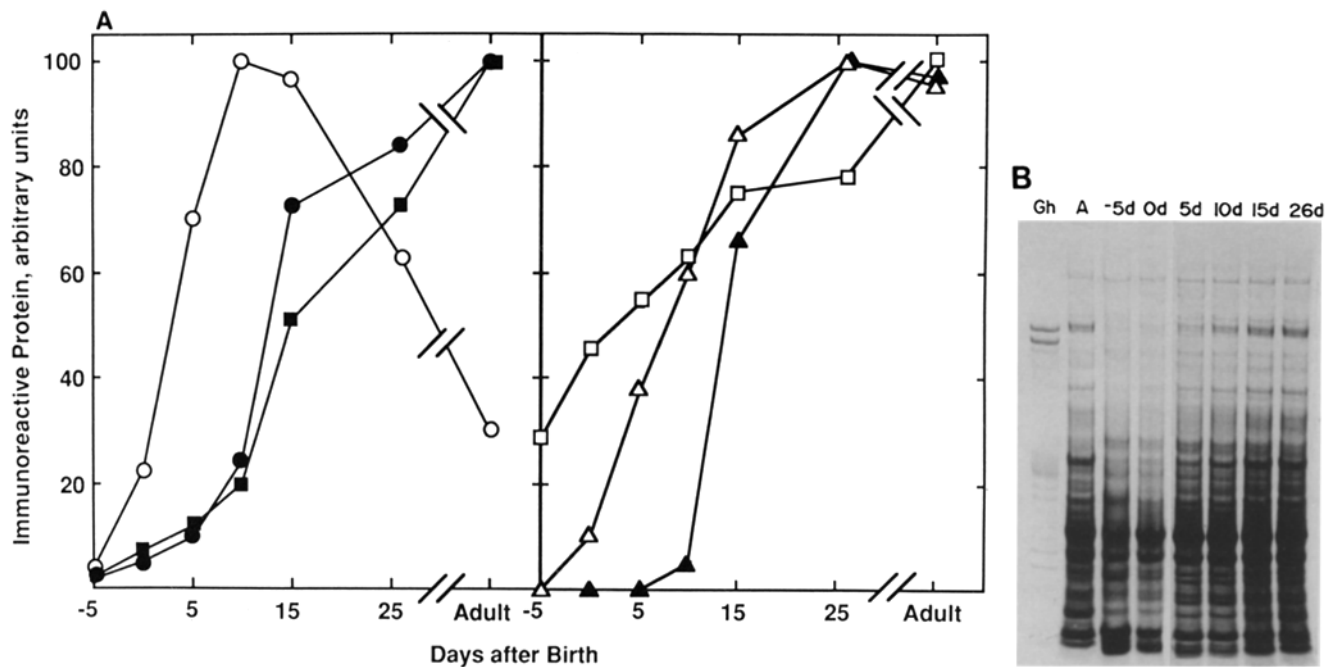


Figure 6. Changes in the amounts of 440 kD (○) and 220-kD ankyrin_B (●), ankyrin_R (■), spectrin (□), synaptophysin (Δ), and myelin basic protein (▲) in developing rat brains. Total homogenate proteins of brains from rats 5 d before birth (-5) and 0, 5, 10, 15, and 26 d after birth and from adult rats (A) were separated on SDS-PAGE, electrophoretically transferred to nitrocellulose filter paper and subjected to immunoblotting. (A) Amounts of proteins reacting with antibody against 440-kD brain ankyrin (●), 220-kD brain ankyrin (■), ankyrin_R (□), bovine brain spectrin (Δ), synaptophysin (▲), and myelin basic protein (▲) were estimated by densitometry of autoradiograms. Data were normalized with respect to amount of protein as determined by scanning a Coomassie blue-stained gel, and are expressed as per cent of the maximal value for each protein. Coomassie blue stained gel pattern is also shown (B). (Gh, human erythrocyte ghost).

clude that the 220 kD ankyrin is encoded by a transcript of 9 and not 13 kb.

The amount of 440-kD ankyrin in adult forebrain was ~15% of the amount of 220-kD ankyrin_B as estimated using antibody against 440/220/150-kD polypeptides (not shown). Since the total amount of ankyrin_B has been estimated to be 0.5–1% of the membrane protein (2, 7), the 440-kD polypeptide comprises ~0.07–0.15% of the membrane protein or 1.5–3 pmols/mg. Spectrin, for comparison, is present in 30 pmols/mg in adult forebrain (7).

Immunoblot analyses of rat brain homogenate proteins also were performed using antibodies against ankyrin_R of human erythrocytes, brain spectrin, synaptophysin and myelin basic protein (Fig. 4, c, d, e, and f, respectively; and Fig. 6). Ankyrin_R and myelin basic protein exhibited developmental changes similar to that of the 220-kD ankyrin_B. Synaptophysin, a marker protein for synaptogenesis, appeared in developing rat brain ~10 d earlier than myelin basic protein, a marker for myelin formation, and both proteins increased sharply within 15 d after birth. Expression of brain spectrin preceded all types of ankyrins and was evident at embryonic day 17 (5 d before birth).

440-kD Ankyrin_B Is A Peripheral Membrane Protein with A General Distribution in Neonatal Rat Brain

440-, 220-, and 150-kD ankyrin_B isoforms are membrane-associated proteins based on the operational definition that they were exclusively present in particulate fractions and absent from cytosolic fraction (200,000 g supernatant) in 10-d-

old and adult rat brains (Fig. 7 A). These ankyrins are peripheral membrane proteins since they were completely solubilized from membranes with 0.1 N NaOH (Fig. 7 B). Moreover, extraction of membranes with nonionic detergent (Triton X-100) released only a portion (<30% from visual inspection of immunoblots) of the 220-kD protein and none of 440-kD protein (Fig. 7 B, lanes 4 and 5). The 150-kD ankyrin_B, in contrast to the larger isoforms, was solubilized completely by extraction with Triton X-100 (Fig. 7 B, lanes 4 and 5).

Relative levels of brain ankyrin isoforms in different parts of rat brain were examined using forebrain, cerebellum and brain stem. The 440- and 220-kD polypeptides were detected in all parts of brains from both 10-d-old and adult rats (Fig. 8). The 150-kD form was expressed in approximately equal amounts in all areas of adult brain except cerebellum, where it was present in low amounts (Fig. 8 b, lane 3). The 440-kD ankyrin_B was expressed in approximately three- to fourfold higher amounts in the 10-d rats compared to adults in total brain and forebrain (Fig. 8) as noted above (Fig. 6). However, 440-kD ankyrin_B is expressed in approximately equal amounts in 10-d and adult cerebellum. The ratio of 440- to 220-kD ankyrin_B in cerebellum is approximately the same as in other regions of the brain due to a robust expression of the 220 kD polypeptide in adult cerebellum.

Cellular Distribution of Ankyrin_B Isoforms in Neonatal and Adult Rat Brain

Ankyrin_B isoforms were localized by indirect immunofluo-

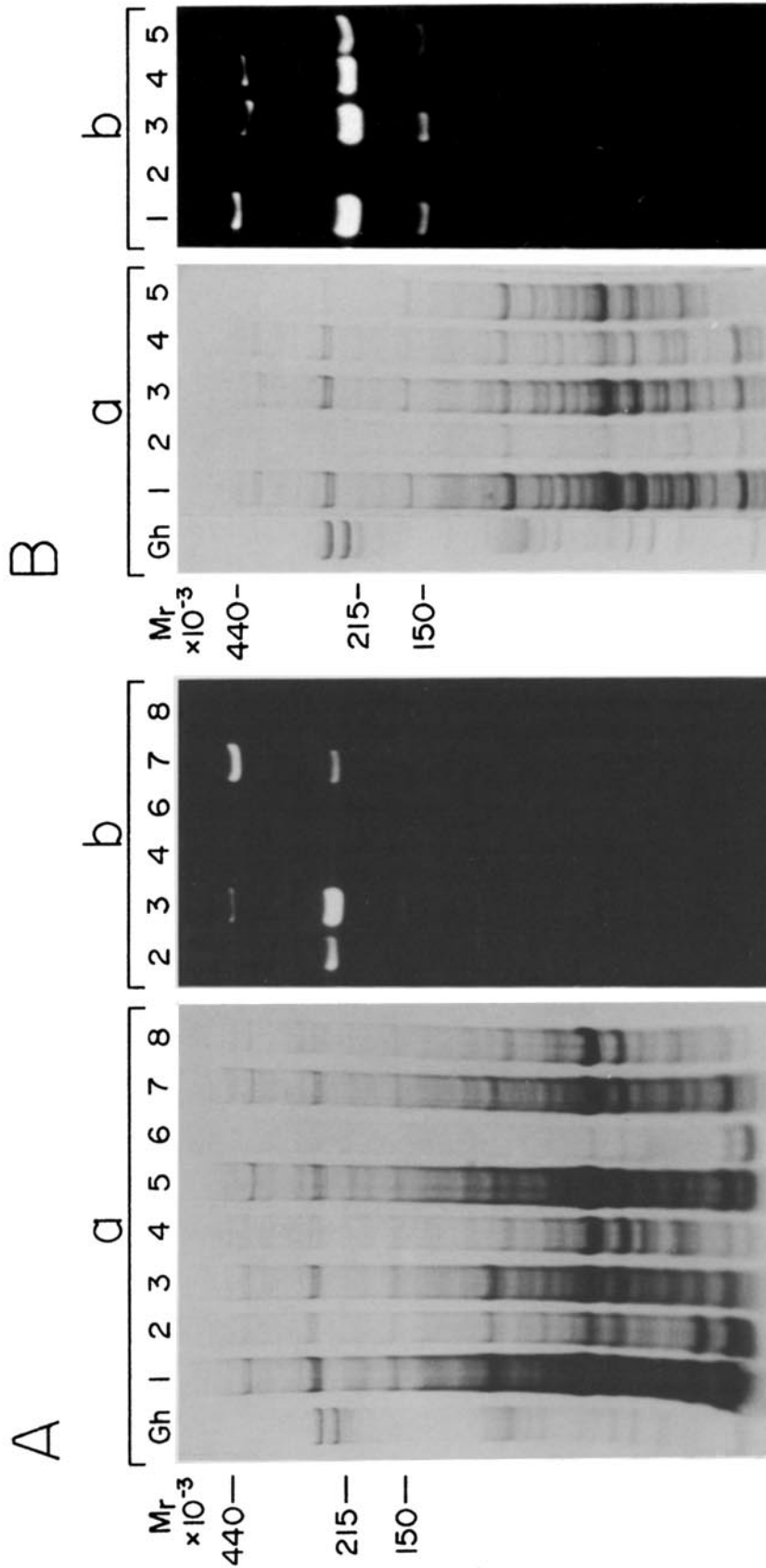


Figure 7. Subcellular fractionation and solubilization of ankyrin_B isoforms. (A) Brain homogenates from adult and 10-d-old rats were centrifuged at 1,000 g for 10 min and the resulting supernatants were centrifuged at 200,000 g for 30 min. Total homogenates (lanes 1 and 5), 1,000 g pellets (lanes 2 and 6), 200,000 g pellets (lanes 3 and 7) and 200,000 g supernatants (lanes 4 and 8) from adult (lanes 1-4) and 10-d-old rat (lanes 5-8) brains were electrophoresed on SDS-PAGE and either stained with Coomassie blue (a) or transferred to nitrocellulose membrane and immunoblotted with antibody against 440/220/150-kD ankyrin_B polypeptides (b). (B) Postnuclear supernatant (1,000 g supernatant) of 26-d-old rat brains was centrifuged at 30,000 g for 25 min. Resulting crude brain membrane fractions were incubated either with 0.1 M NaOH (lanes 2 and 3) or with 1% Triton X-100 (lanes 4 and 5) for 30 min at 4°C and then centrifuged at 100,000 g for 30 min. Pellets (lanes 3 and 4), supernatants (lanes 5 and 6), and crude membrane fraction (lane 7) were electrophoresed on SDS-polyacrylamide gels and either stained with Coomassie blue (a) or transferred to nitrocellulose filter paper and immunoblotted with antibody against 440/220/150-kD ankyrin_B polypeptides (b).

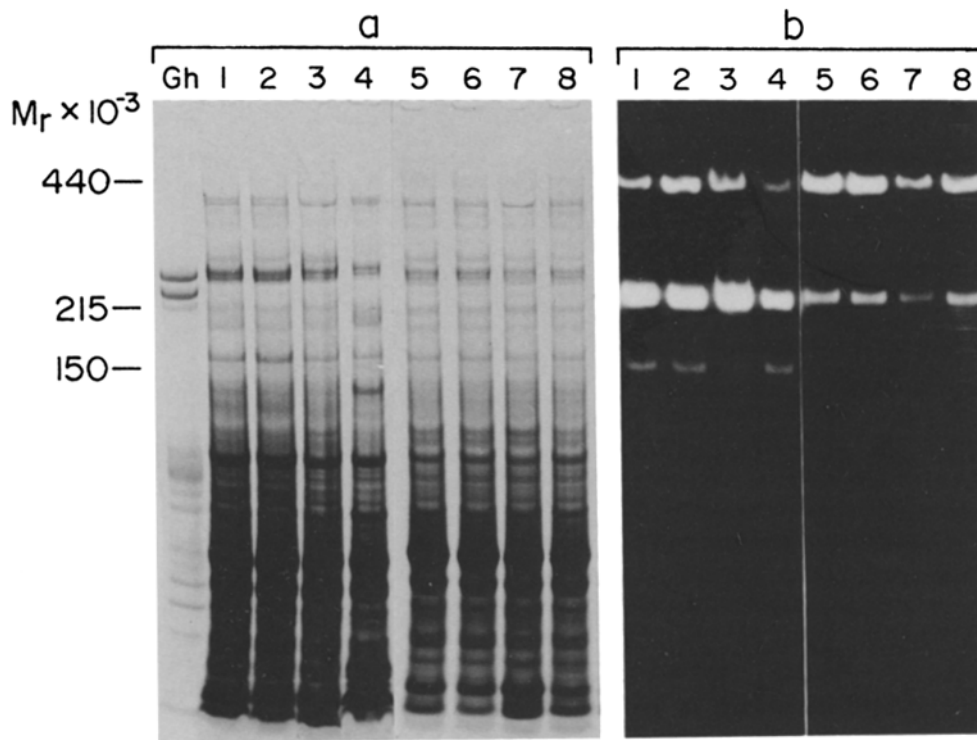


Figure 8. Regional distribution of ankyrin_B isoforms in rat brain. Homogenates were prepared from whole brain (lanes 1 and 5), forebrain (lanes 2 and 6), cerebellum (lanes 3 and 7) and brain stem (lanes 4 and 8) of adult (lanes 1-4) and 10-d-old (lanes 5-8) rats, electrophoresed on SDS-polyacrylamide gels and either stained (a) or transferred to nitrocellulose filter paper and immunoblotted with antibody against 440/220/150-kD ankyrin_B polypeptides (b).

rescence in cryosections of cerebellum from 10-d-old and adult rats using antibodies described above that are specific for either the 440-kD polypeptide or which cross-react with the 440, 220, and minor 150-kD ankyrin_B polypeptides. Fig. 9 shows the comparison of serial sections stained either with Multi-stain (b) or with antibody against 440/220/150-kD polypeptides visualized by indirect immunofluorescence (a). The molecular layers of both adult and neonatal cerebellum were highly labeled in a general pattern by this antibody (Fig. 9, a and c). The molecular layer of adult rats begins at the surface and extends inward to the layer of Purkinje neuron cell bodies while in neonatal animals the molecular layer is a narrow zone located between inner and an outer zones of granule cells. In both adults and neonatal animals major components of the molecular layer of cerebellum are bundles of unmyelinated axons known as parallel fibers that originate from granule cells, and the dendrites of Purkinje cells. The parallel fiber axons are involved in extensive synaptic contacts with Purkinje cell dendrites (25). Therefore the extensive labeling of the molecular layer suggests that ankyrin is present in unmyelinated axons and could also be present in dendrites of Purkinje cells. However, the label is not confined to Purkinje cell dendrites since in this case a characteristic arborized branching pattern would result rather than the generalized staining actually observed.

The 440-kD polypeptide in adult cerebellum is exclusively localized in the molecular layer and is absent from Purkinje cell bodies as well as granule cells (Fig. 10). Antibody recognizing 440/220/150-kD polypeptides, in contrast, labeled Purkinje cell bodies and granule cells in addition to the molecular layer (Fig. 9, inset). Brain spectrin also was detected in Purkinje cell bodies and granule cells as well as the molecular layer. 440-kD ankyrin_B thus has a more restricted distribution than other forms of ankyrin_B, and is highly polarized to locations in neuronal processes.

440 kD ankyrin_B exhibits a similar restricted pattern in neonatal cerebellum, where it is concentrated in the developing molecular layer and absent from Purkinje cell bodies (Fig. 10). In contrast to the adult cerebellum, some label is detectable deeper in the granule layer in an extended fibrillar pattern. It is not possible to identify cellular structures corresponding to this label at the level of light microscopy. However, one possibility is that the fibers represent bundles of unmyelinated axons. Antibody against the 440/220/150-kD ankyrins provided a similar pattern of label as the antibody selective for the 440-kD polypeptide, as would be expected since the 440-kD ankyrin is the predominant form at this stage of development. However, some staining of Purkinje cell bodies and of the outer layer of granule cells can be detected with antibody against 440/220/150-kD ankyrins which is absent in sections stained with antibody selective for the 440-kD form of ankyrin (Fig. 10). Spectrin was present in a pattern similar to that of the 440/220/150-kD ankyrin_B polypeptides (Fig. 10).

The cerebral cortex and hippocampus in neonatal forebrain contain acellular zones composed of unmyelinated axons and dendrites analogous to the molecular layer of the cerebellum. 440-kD ankyrin_B is highly concentrated in these areas by immunofluorescence (Figs. 9 and 11). Staining also is present among the cell bodies of neurons. It is not possible at this level of resolution to determine if 440-kD ankyrin_B is located on neuronal cell bodies, or is present in axons and dendrites of other neurons that are known to pass through this area. The staining exhibits a punctate pattern both in the outer layer and between neuronal cell bodies that suggests an elevated concentration at synapses.

Discussion

This report describes initial characterization of a novel 440-

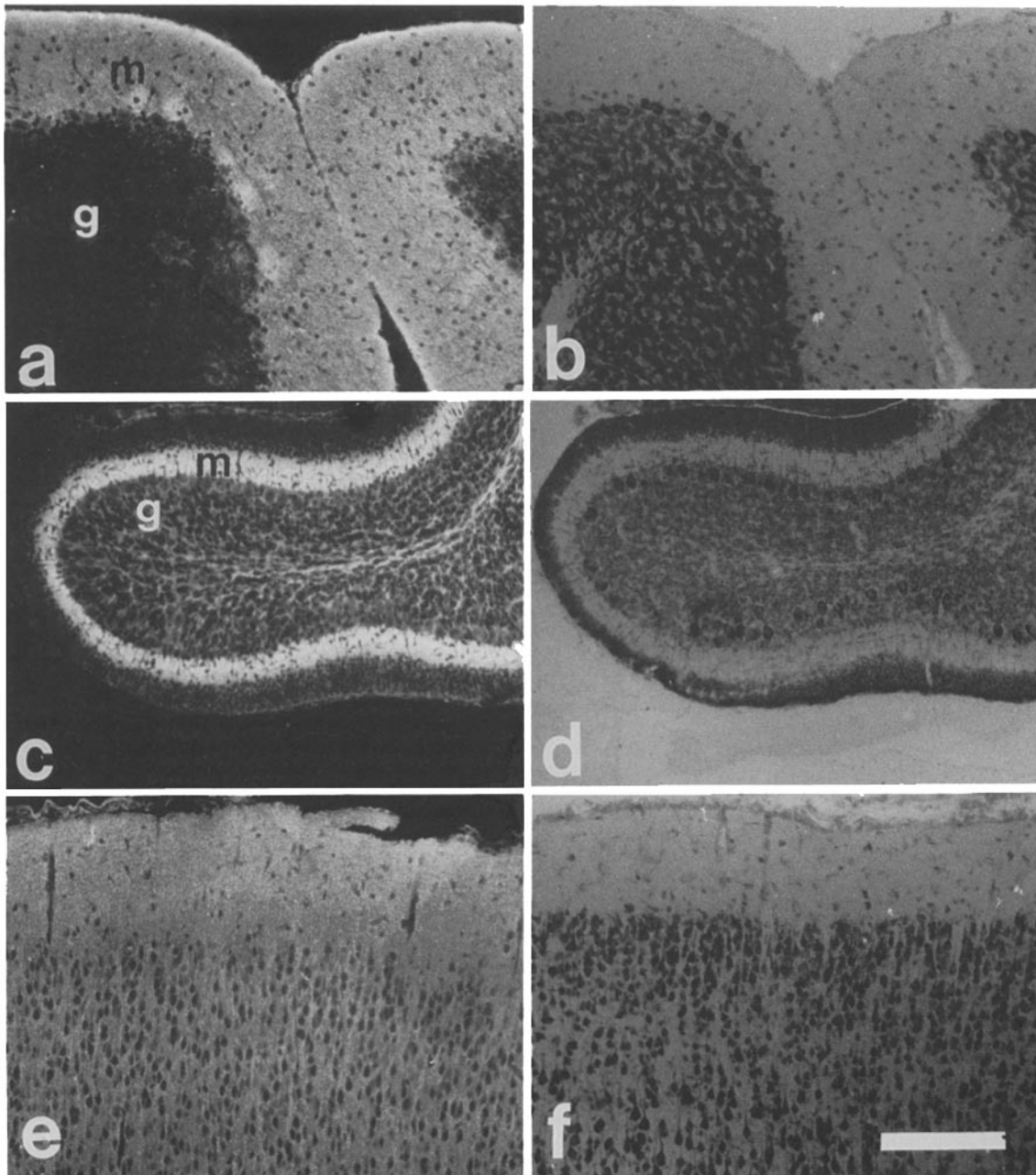


Figure 9 Immunocytochemical detection of ankyrin_B isoforms in neonatal (*c* and *d*) and adult (*a* and *b*) cerebellum and neonatal cerebral cortex (*e* and *f*) of rat brain. 10 μ m cryosections of cerebellum (*a*–*d*) and forebrain (*e* and *f*) of adult (*a* and *b* and *e* and *f*) and 10-d-old (*c* and *d* and *g* and *h*) rats were labeled by indirect immunofluorescence with antibody against 440/220/150-kD ankyrin_B polypeptides (*left column*) or stained with Multistain dye solution (*right column*). The location of the molecular layer is noted by an *m* and that of the granule layer with a *g* in *A* and *C*. Bar, 100 μ m.

kD isoform of brain ankyrin (ankyrin_B) discovered as the result of analysis of cDNA clones encoding the major adult form of human brain ankyrin (24). Northern and immunoblot analyses indicate that the 440-kD ankyrin_B includes the spectrin and membrane-binding domains as well as a predicted regulatory domain of the major 220-kD isoform. 440-kD ankyrin_B contains, in addition, a sequence of a predicted size of 220 kD which is inserted between the regulatory domain and spectrin/membrane-binding domains (Fig. 1). 440-kD ankyrin_B has properties expected of a peripherally associated membrane-skeletal protein: it is ex-

clusively present in the particulate fraction of brain homogenates, is extracted with NaOH, and remains associated with Triton-X-100-resistant structures. 440-kD ankyrin_B is the major form of ankyrin that can be detected in developing rat brain, and begins to appear at birth. Expression of 440-kD ankyrin_B is maximal in the neonatal period of rat development, with a peak at day 10, preceding myelin basic protein and synaptophysin. 440-kD ankyrin_B persists in adults, although the pattern of expression is less general than in neonatal brain tissue. Adult cerebellum contains nearly the same amount as neonatal cerebellum, while very low expression

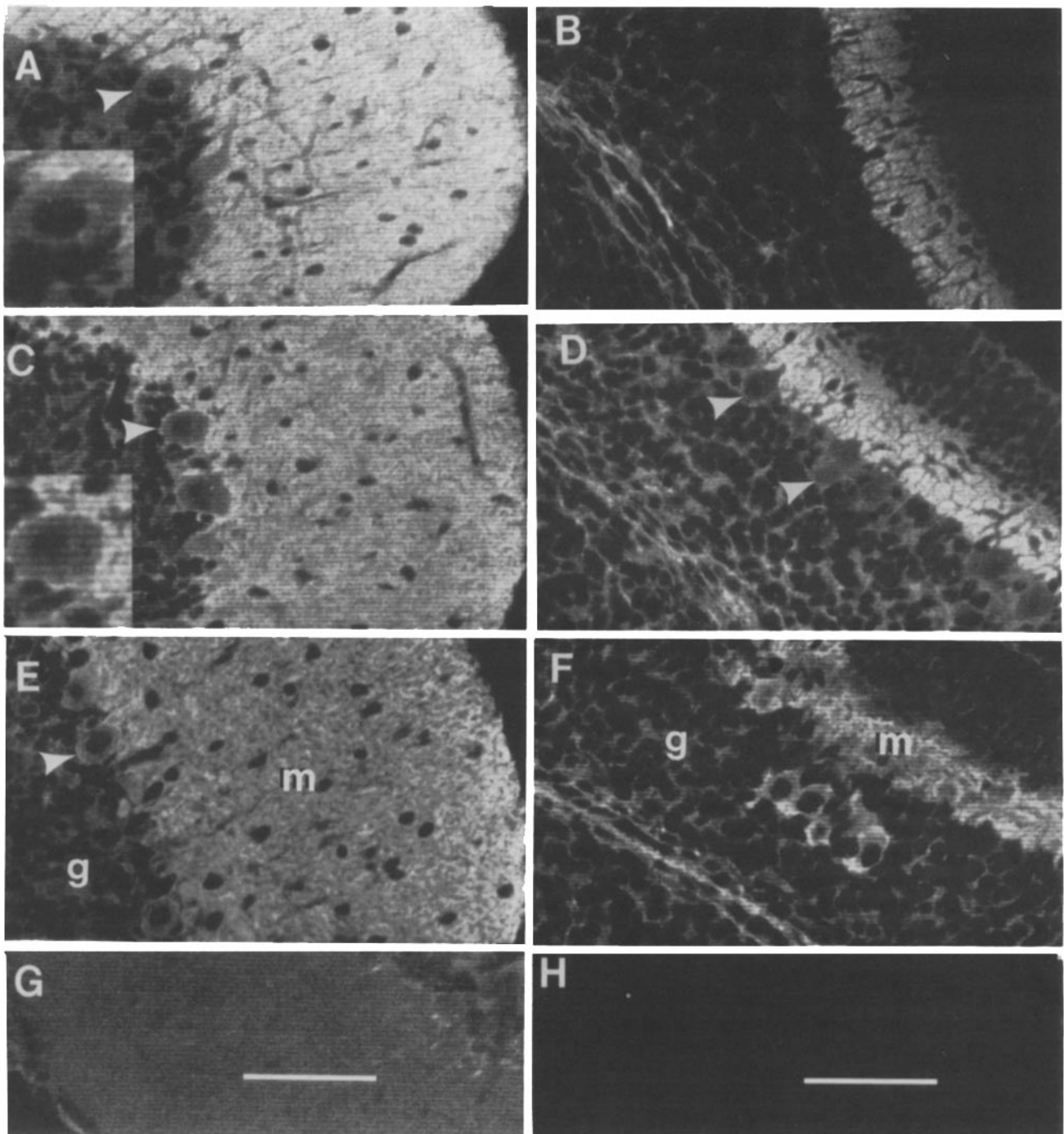


Figure 10. Localization of ankyrin_B isoforms in gray matter of adult and neonatal rat cerebellum. 10 μ m cryosections of cerebellum of adult (A, C, E, and G) and 10-d-old (B, D, F and H) rats were labeled by indirect immunofluorescence with antibodies against 440-kD ankyrin_B (a and b), 440/220/150-kD ankyrin_B polypeptides (c and d), brain spectrin (e and f), or control IgG (g and h), and visualized with a confocal microscope. Arrows indicate Purkinje cell bodies. Purkinje cell bodies (a and c, insets) are not stained by antibody against 440-kD ankyrin_B but do react with antibody against 440/220/150-kD ankyrins. The locations of the molecular layer (m) and granule layer (g) are marked in E and F. Bar, 50 μ m.

of 440-kD ankyrin_B occurred in adult brain stem. 440-kD ankyrin_B was localized by immunofluorescence in molecular layers of cerebellum, cerebral cortex, and hippocampus, which are regions comprised mainly of unmyelinated axons and dendrites with relatively few glial cells or neuronal cell bodies.

Conclusions from the morphology are that 440-kD an-

kyrin_B is expressed by neurons and is targeted to neuronal processes which may be either in axons and/or dendrites. 440-kD ankyrin_B is highly polarized in its distribution since it is absent from cell bodies of cerebellar neurons known to be major contributors of axons and dendrites to the molecular layer. Other brain ankyrin isoforms are present in these neuronal cell bodies, suggesting that confinement to neuronal

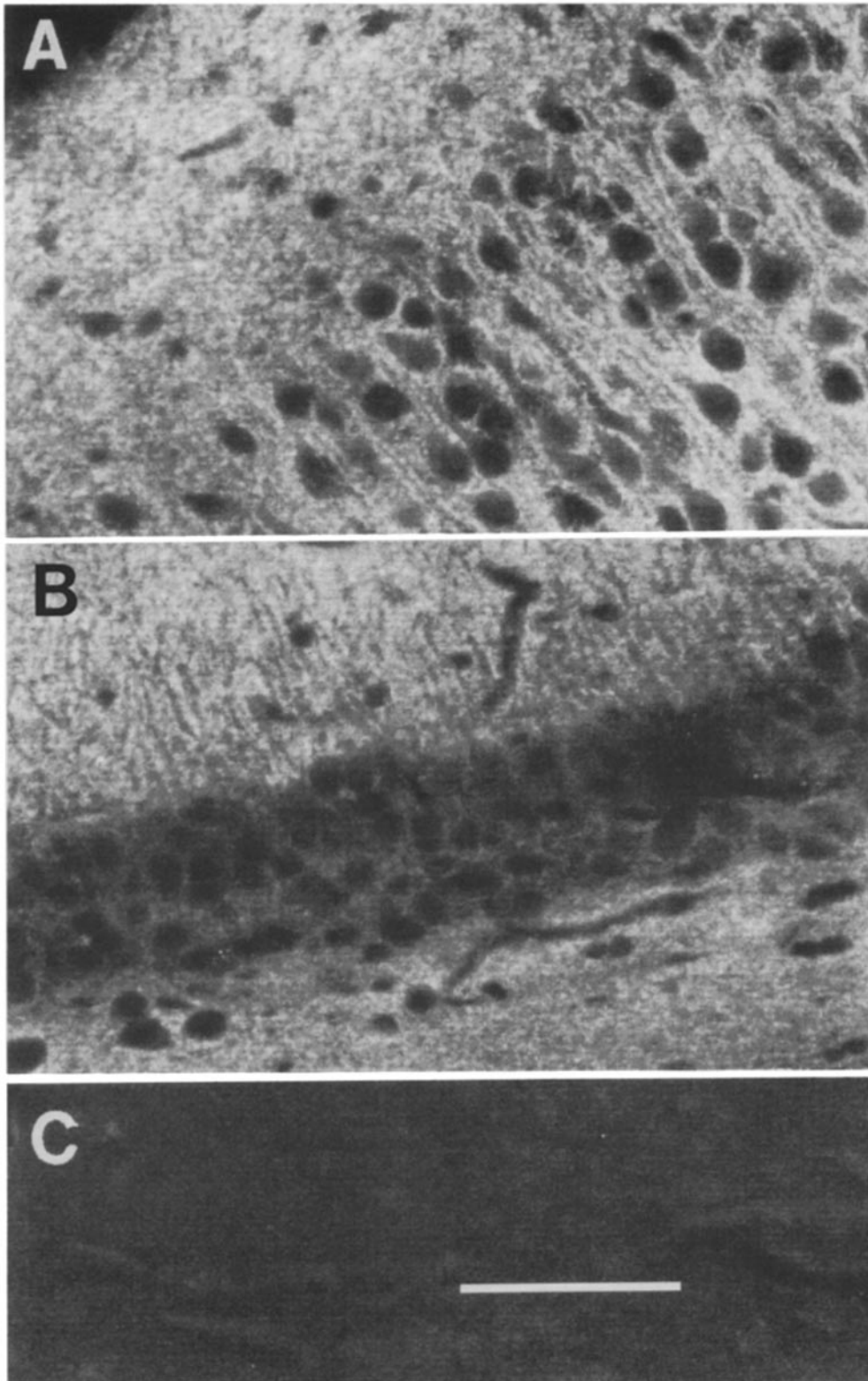


Figure 11. Localization of the 440-kD ankyrin_B isoform in molecular layers of neonatal rat cerebral cortex (A) and hippocampus (B). 10 μm cryosections were prepared from brains of 10-d-old rats and incubated with antibody against 440-kD ankyrin_B as described (Fig. 9). C is a section of neonatal cortex incubated with control Ig. Bar, 50 μm.

processes is a special feature of the 440-kD isoform. A more precise localization of 440-kD ankyrin_B will require EM. Evidence that at least some of the label is present in unmyelinated axons is provided by the intense staining of the molecular layer in the 10-d cerebellum, since at this stage the Purkinje cell dendrites are undeveloped and the molecular layer

contains bundles of unmyelinated axons (1). Moreover, preferential localization of 440-kD ankyrin_B in unmyelinated axons is consistent with low expression in regions of the brain such as brain stem which are enriched in myelinated axons and would explain the generally lower levels of 440-kD ankyrin_B in adult brain tissue.

The presence of 440-kD ankyrin_B in neuronal processes and its high expression during a relatively short period of development suggest that this isoform performs a specialized role in brain. Possibilities include functions related to particular requirements of unmyelinated axons such as physical support of their plasma membranes, stabilization of contacts with adjacent axons in the bundles that can be visualized by EM (25), and in formation of multiple synapses with dendrites. The sequence that is unique to the 440-kD isoform presumably is required either for preferential targeting and/or for a specialized role once localization has been achieved. It will be important in future work to elucidate the complete sequence of 440-kD ankyrin and evaluate the functional properties of the additional regions. One interesting feature of this sequence that already is known is a series of 15 repeats of 12 residues with the consensus of: HPPVSPS-KTER (24).

Expression of ankyrins in avian neuronal cells have been previously noted to be developmentally regulated (22). In chick brain, a brain-type ankyrin is first expressed at the embryonic stage and replaced by erythrocyte-type ankyrin. These ankyrins had apparent molecular weights in the range of 200 kD, and presumably are distinct from the 440-kD isoform described in this study. It is difficult to compare details of brain development of chickens, which at birth already are highly developed, with species such as rats or humans which experience significant further development in the neonatal period.

440-kD ankyrin_B provides an additional example of significant diversity within the ankyrin family of proteins. Brain tissue expresses products of at least two distinct ankyrin genes, the *ANK1* gene that encodes ankyrin_R of erythrocytes and is located on chromosome 8 (18, 19) and *ANK2* gene encoding ankyrin_B isoforms that is located on chromosome 4 (24). Moreover, studies with mutant mice missing ankyrin_R suggest a third ankyrin gene product that is localized at nodes of Ranvier of myelinated nerves (16). Additional diversity has been shown to result from alternative splicing of pre-mRNAs encoded by these ankyrin genes (18, 20, 24). Alternative forms of *ANK1* include a variant (protein 2.2 in the RBC membrane nomenclature) that is missing a portion of an exon in the regulatory domain, and is an activated form of ankyrin (10, 14, 20). Another splicing event involves a highly basic stretch of 32 residues (pI >10) located at the COOH-terminus of the regulatory domain (18). The *ANK2* gene appears to encode 13- and 9-kb mRNAs that are responsible for the 440-kD isoform and major 220-kD brain ankyrin, respectively. Northern blots of brain mRNA reveal an additional mRNA of 6 kb that hybridized with probes derived from sequence corresponding to the proposed regulatory domain as well as spectrin-binding domain of the 220-kD brain ankyrin. However, the 6-kD mRNA did not hybridize with probes corresponding to the membrane-binding domain, and presumably encodes a polypeptide missing a membrane-binding domain. A polypeptide of 150 kD was detected by antibody against the regulatory domain that is a candidate to be the protein encoded by the 6-kb mRNA. The 150-kD polypeptide, in contrast to other brain ankyrin isoforms, is solubilized by extraction with Triton X-100 (Fig. 7). The existence of an ankyrin capable of associating with spectrin but missing the membrane-binding domain stretches the concept of diversity to a point requiring re-evaluation of potential functions of the ankyrin family.

The two currently known ankyrin genes are likely to have evolved by a duplication event following development of spectrin-binding and the 33-residue repeats of the membrane-binding domains. It is not known at this time if both of these genes also share the inserted sequence present in 440-kD ankyrin_B. Northern blots of adult tissues with *ANK1* probes have not yet revealed a mRNA >9 kb, suggesting the possibility that the additional sequence is a special feature of the *ANK2* gene which was either inserted or lost during evolution. It will be of interest in the future to determine if the inserted sequence is comprised of a single exon, and to elucidate the evolutionary relationship between *ANK1* and *ANK2* genes.

Expression of spectrin preceded ankyrins during development in rat brain (Fig. 6). Although the presence of an unidentified early isoform of ankyrin cannot be excluded, it also is possible that initial assembly of a spectrin skeleton on the membrane could result from association of spectrin with membranes via ankyrin-independent linkage(s) such as recently described in brain membranes (28, 29). In this case, ankyrins would perform functions required in differentiated cells rather than a constitutive role. Specialized activities for ankyrins are also implied by the degree of variability within this family noted above.

In conclusion, at least two isoforms of ankyrin_B of 220 and 440 kD are expressed in rat brain that are developmentally regulated and are likely to result from alternative processing of mRNA. The 440-kD isoform is highly expressed in the neonatal period in regions of brain containing primarily unmyelinated axons which are in the process of developing multiple axodendritic synapses. The 440-kD ankyrin thus may play a role in the formation of neuronal networks in developing brain.

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