Ankyrin-binding Proteins Related to Nervous System Cell Adhesion Molecules: Candidates to Provide Transmembrane and Intercellular Connections in Adult Brain

Jonathan Q. Davis, Tanya McLaughlin, and Vann Bennett

Howard Hughes Medical Institute and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Abstract. A major class of ankyrin-binding glycoproteins have been identified in adult rat brain of 186, 155, and 140 kD that are alternatively spliced products of the same pre-mRNA. Characterization of cDNAs demonstrated that ankyrin-binding glycoproteins (ABGPs) share 72% amino acid sequence identity with chicken neurofascin, a membrane-spanning neural cell adhesion molecule in the Ig super-family expressed in embryonic brain. ABGP polypeptides have the following features consistent with a role as ankyrin-binding proteins in vitro and in vivo: (*a*) ABGPs and ankyrin associate as pure proteins in a 1:1 molar stoichiometry; (*b*) the ankyrin-binding site is located in the COOH-terminal 21 kD of ABGP186 which contains the predicted cytoplasmic domain; (*c*) ABGP186 is expressed at approximately the same levels as ankyrin (15 pmoles/milligram of membrane protein); and (*d*) ABGP polypeptides are co-expressed with the adult form of ankyrin$_B$ late in postnatal development and are colocalized with ankyrin$_B$ by immunofluorescence. Similarity in amino acid sequence and conservation of sites of alternative splicing indicate that genes encoding ABGPs and neurofascin share a common ancestor. However, the major differences in developmental expression reported for neurofascin in embryos versus the late postnatal expression of ABGPs suggest that ABGPs and neurofascin represent products of gene duplication events that have subsequently evolved in parallel with distinct roles. The predicted cytoplasmic domains of rat ABGPs and chicken neurofascin are nearly identical to each other and closely related to a group of nervous system cell adhesion molecules with variable extracellular domains, which includes L1, Nr-CAM, and Ng-CAM of vertebrates, and neuroglian of Drosophila. The ankyrin-binding site of rat ABGPs is localized to the C-terminal 200 residues which encompass the cytoplasmic domain, suggesting the hypothesis that ability to associate with ankyrin may be a shared feature of neurofascin and related nervous system cell adhesion molecules.

PROTEIN–protein linkages extending across the plasma membrane between extracellular and intracellular compartments are required for maintenance of specialized cell surface domains in metazoan cells and organization of these cells into tissues. Resolution of details of the interacting proteins involved in transmembrane associations is especially challenging in vertebrate brain with its diversity of cell domains and cell types and intricate cellular organization. Nevertheless a solution to this question will provide fundamental insights into mechanisms of development and maintenance of the cellular architecture of the nervous system.

Ankyrins are a family of structural proteins strategically located on the cytoplasmic surface of the plasma membrane with recognition sites for both membrane-spanning integral membrane proteins and cytoplasmic structural proteins (reviewed by Bennett, 1990, 1992). Ankyrins are ancient components of the nervous system that are expressed in *Caenorhabditus elegans* (Otsuka, A. J., R. Franco, B. Yang, K.-H. Shim, L. Z. Tang, A. Jeyaprakash, and P. Boontrakulpoontawee. 1991. *J. Cell Biol.* 115:465a) and comprise 0.5–1% of the membrane protein in adult vertebrate brain (Bennett, 1979; Davis and Bennett, 1984). Multiple isoforms of ankyrin are expressed in brain with diversity due to distinct genes as well as alternative splicing of mRNAs: 220-kD ankyrin$_B$, which is generally distributed in neurons and glial cells of adult brain; 440-kD ankyrin$_B$, an alternatively spliced form highly expressed in neonatal development and located in neuronal processes; 215-kD ankyrin$_R$, which is confined to cell bodies and dendrites of a subset of neurons; and ankyrin$_{node}$, localized at axonal initial segments and nodes of Ranvier. A common feature of the ankyrin family is a membrane-binding domain comprised of 22 repeats of a 33-residue motif implicated in macromolecular recognition in a variety of proteins (reviewed by Michaely and Bennett, 1992).

Initial characterization of membrane-binding sites for ankyrin in mammalian brain revealed a class of integral membrane proteins capable of high affinity association with the membrane-binding domain of ankyrin$_B$, which are pres-

ent in amounts comparable with ankyrin (Davis and Bennett, 1986). Membrane proteins demonstrated to associate with ankyrin as pure proteins and colocalized with ankyrin in tissues include the voltage-dependent sodium channel (Srinivasan et al., 1988; Kordeli et al., 1990; Srinivasan et al., 1992), and the Na/K ATPase (Nelson and Veshnock, 1987; Koob et al., 1987; Morrow et al., 1989). In addition, other proteins that are candidates to associate with ankyrin include CD44 (Kalomiris and Bourguignon, 1988), and ABGP205 (Treharne et al., 1988).

This report describes isolation of a major class of ankyrin-binding membrane glycoproteins (ABGPs)[1] from adult rat brain, using as an affinity adsorbent the 33-residue repeat domain of ankyrin$_B$ expressed in bacteria. cDNA cloning of these proteins reveals that they are closely related to chicken neurofascin, a membrane-spanning cell adhesion molecule of the Ig super family implicated in axonal bundling in development of embryonic chicken brain (Rathjen et al., 1987; Volkmer et al., 1992). The predicted cytoplasmic domains of rat ankyrin-binding proteins and chicken neurofascins are nearly identical to each other and closely related to a group of nervous system cell adhesion molecules with variable extracellular domains, which includes L1 (Moos et al., 1988), Nr-CAM (Grumet et al., 1991), and Ng-CAM (Burgoon et al., 1991) of vertebrates, and neuroglian of *Drosophila* (Bieber et al., 1989). The ankyrin-binding site of rat ABGPs is localized to the COOH-terminal 200 residues which encompass the cytoplasmic domain, suggesting the possibility that ability to associate with ankyrin may be a shared feature of neurofascin and related nervous system cell adhesion molecules.

Materials and Methods

Methods

SDS-PAGE, immunoblot analysis after transfer of polypeptides to nitrocellulose paper, low-angle rotary shadowing of proteins with platinum/carbon for EM, preparation of affinity-purified rabbit antibodies, radiolabeling of ankyrin$_B$ (residues 190–942) with ^{125}I-Bolton Hunter reagent, and blot binding with ^{125}I-labeled ankyrin (residues 190–940) were performed as described for ankyrin and other proteins (Davis and Bennett, 1984). Molecular biology methods were performed essentially as described (Sambrook et al., 1989). cDNA clones were subcloned from λ phage into Bluescript (KS+) plasmids. DNA sequencing of plasmids was performed by the dideoxy chain termination method using oligonucleotide primers to initiate the reactions. Protein microsequencing was performed using polypeptides transferred from SDS-gels to immobilon paper as described (Matsudaira, 1987; Davis and Bennett, 1990). Immunocytochemistry with frozen sections of rat tissue from animals perfused with 2% paraformaldehyde was performed as described (Kordeli et al., 1990; Kordeli and Bennett, 1991).

Ankyrin$_B$-Affinity Column

Ankyrin$_B$ (residues 190–942) with three additional residues (met-ala-ser) at the NH_2 terminus was expressed in *Escherichia coli* after ligation of the coding sequence into a pET plasmid containing a T7 promoter (Studier et al., 1990; Davis et al., 1991), and purified from the soluble bacterial extract by gel filtration on a Superose 12 column followed by cation exchange chromatography on S-Sepharose (see Fig. 1, lane *1* for purified protein). Ankyrin was coupled to agarose by addition of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) to an equal volume of solution containing 2 mg/ml of expressed ankyrin, 50 mM sodium phosphate, 0.5 M NaCl, 1 mM NaEGTA, 0.5 mM dithiothreitol, 1 mM Na azide, pH 8.5. After 14 h at 4°C the gel was packed into a column, washed with 20 vols of 1 M NaBr, 0.05% Tween 20, 0.5 mM DTT, 1 mM NaEGTA, 10 mM sodium phosphate, pH 7.4, and then equilibrated with loading buffer: 0.1 M KCl, 10 mM Hepes, 0.5% Triton X-100, 0.01% phosphatidylcholine, 1 mM Na azide, 0.5 mM DTT, pH 7.4.

1. *Abbreviation used in this paper*: ABGP, ankyrin-binding membrane glycoprotein.

Preparation of Triton X-100 Extracts of Brain Membranes

Rat brain tissue (obtained from Pel-Freez Biologicals, Rogers, AK) was homogenized with a Brinkman Polytron in 10 vols of 0.32 M sucrose, 2 mM NaEGTA, 1 mM DTT, 5 mM Tris-HCL, pH 7.4, with protease inhibitors: leupeptin (5 μg/ml); pepstatin A (5 μg/ml), PMSF (100 μg/ml), and diisopropylfluorophosphate (0.5 mM). The homogenate was centrifuged for 10 min at 900 *g* to remove nuclei and remaining tissue fragments, and the 900 *g* supernatant centrifuged for 1 h at 100,000 *g*. The pellet was resuspended in homogenization buffer, recentrifuged, and extracted for 30 min on ice with 7 vols (based on the initial amount of tissue) of extraction buffer (2.5% Triton X-100, 0.1% phosphatidylcholine, 0.1 M KCl, 10 mM Hepes, 2 mM DTT, 1 mM Na azide, pH 7.4). The suspension was centrifuged for 45 min at 100,000 *g* and the supernatant used for purification procedures.

Immobilization of Glycoproteins for Ankyrin-binding Assays

Ankyrin-binding glycoproteins were immobilized through their carbohydrate residues on the surface of Concanavalin A-beads (Affigel 701 amine-derivatized polyacrylamide beads 1–3 μm in diameter with 9-kD exclusion; BioRad Labs., Hercules, CA). Glycoprotein-coated beads could then be rapidly separated from free ankyrin in ankyrin-binding assays by sedimentation through a glycerol barrier gradient. Affigel 701 beads (5 ml of a 10% suspension) were incubated for 14 h at 4°C with 25 mg of Concanavalin A and 20 mg of the water-soluble carbodiimide EDAC (1-Ethyl-3[3-dimethylamino-propyl] carbodiimide) in coupling buffer: 3 mM potassium phosphate, 1 mM each of calcium chloride, manganese chloride, and sodium azide, pH 6.6. The beads were pelleted and resuspended four times with washing buffer: 10 mM Hepes, 0.2% Triton X-100, 0.15 M NaCl, 0.5 mM DTT, and 1 mM each of calcium chloride, manganese chloride, sodium azide, pH 7.4, and resuspended at a 10% suspension in the same buffer. Concanavalin A-beads were incubated with isolated glycoproteins for 14 h at 4°C in wash buffer, pelleted, and resuspended twice in this buffer, and finally resuspended at a 10% suspension in binding assay buffer: 0.1 M NaCl, 10 mM Hepes, 0.2% Triton X-100, 0.5 mM DTT, 1 mM each of calcium chloride, manganese chloride, sodium azide, and 10 mg/ml of BSA, pH 7.4.

Results

Identification of Ankyrin-binding Membrane Glycoproteins in Adult Rat Brain

Membrane glycoproteins capable of direct association with ankyrin were identified using the following strategy. The membrane-binding domain of ankyrin$_B$ (residues 190–947 comprising repeats 5–22 plus a portion of the spectrin-binding domain) was expressed in bacteria (Davis et al., 1991) and used to prepare an affinity adsorbent. Proteins from detergent extracts of brain membranes were adsorbed to the ankyrin-affinity column, resulting in selection of at least 10 polypeptides (Fig. 1, lane *5*). Glycoproteins in this group of ankyrin-binding polypeptides were isolated using a WGA-affinity column and were eluted with *N*-acetylglucosamine (Fig. 1, lane *6*). Finally, a 186-kD polypeptide capable of direct association with ankyrin was identified in the eluate from the WGA-affinity column by blot-binding with ^{125}I-labeled ankyrin (Fig. 1, *right panels*). Association of the 186-kD polypeptide with ankyrin was selective in that other polypeptides in brain membranes did not bind, and binding was displaced by a 20-fold excess of unlabeled ankyrin.

The 186-kD polypeptide was isolated in sufficient quantities to prepare affinity-purified polyclonal antibodies using sequential ankyrin and WGA-affinity columns followed by

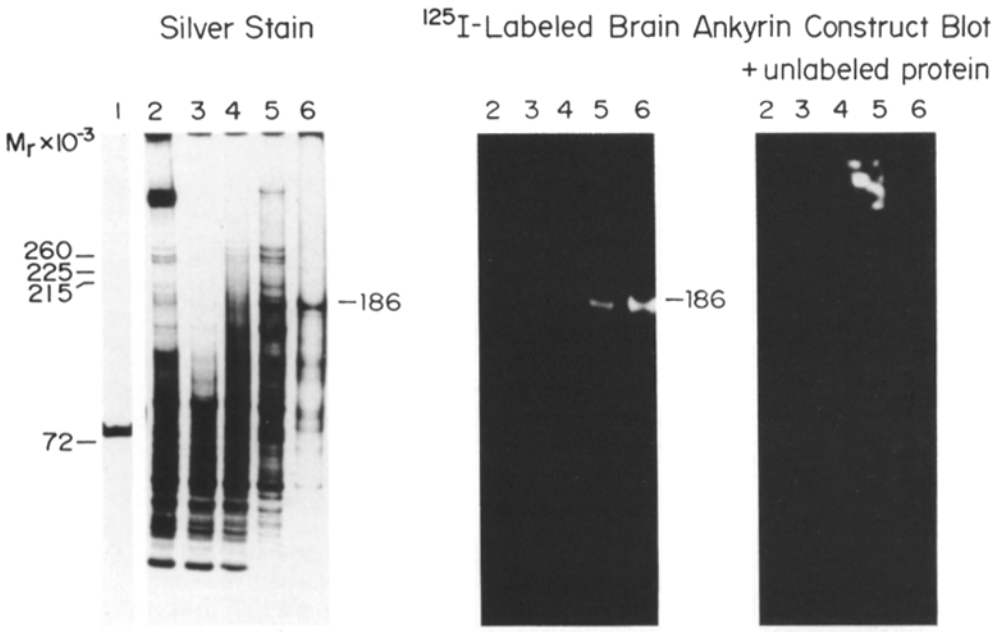


Figure 1. Identification in adult rat brain of a 186-kD membrane glycoprotein with ankyrin-binding activity. Triton X-100–solubilized membrane proteins from 10 g of adult rat brain were applied at 4°C to an ankyrin$_B$ (residues 190–947) affinity column, and the column washed with 10 column volumes of 0.1 M KCl dissolved in column buffer (0.5% Triton X-100, 0.01% phosphatidylcholine, 10 mM Hepes, 2 mM DTT, 1 mM Na azide, pH 7.4). The adsorbed proteins were eluted with 1 M NaBr in column buffer and applied to a WGA–agarose affinity column, which was washed with 10 vols of loading buffer, and adsorbed proteins eluted with 0.2 M *N*-acetyl glucosamine. Peak fractions were analyzed by SDS–electrophoresis, and polypeptides detected either by silver stain (*left*) or by blot-binding with 10 nM ^{125}I-labeled ankyrin$_B$ (residues 190–947) after electrophoretic transfer of polypeptides to nitrocellulose paper (see Materials and Methods) (*right panels*). A control blot was performed with a 20-fold excess of unlabeled ankyrin (*far right panel*). (Lane *1*) Coomassie blue–stained gel of ankyrin$_B$ (residues 190–947) expressed in *E. coli* and purified as described (see Materials and Methods); (lane *2*) total rat brain membranes; (lane *3*) Triton X-100 extract of brain membranes; (lane *4*) Triton X-100 extract after passage over the ankyrin-affinity column; (lane *5*) polypeptides eluted from the ankyrin-affinity column; and (lane *6*) polypeptides eluted from the wheat germ affinity column.

ion exchange on a Mono S column (Fig. 2*a*). Direct evidence that the 186-kD polypeptide is glycosylated is provided by reduction in M_r to 180 kD after digestion with endoglycosidase H and to 165-kD by digestion with endoglycosidase F (Fig. 2*b*).

The 186-kD ankyrin-binding glycoprotein (ABGP186) is relatively abundant in adult brain tissue, based on quantitative immunoblots. (Fig. 3). ABGP186 comprises ~0.3% of the total membrane protein, corresponding to 15 pmoles per milligram of membrane protein. For comparison, amounts of ankyrin in adult brain have been estimated to be in the range of 10–50 pmoles per milligram, and ankyrin-binding sites at 25 pmoles per milligram (Davis and Bennett, 1984, 1986). ABGP186 is therefore present in sufficient amounts to account for a major portion of the ankyrin-binding proteins in adult brain membranes.

ABGP186 Is a Member of a Family of Sequence-related Proteins

Affinity-purified Ig isolated using ABGP186 as an immunogen and as an affinity adsorbent also cross reacts with two polypeptides of apparent M_r of 155 and 140 kD in immunoblots of brain membranes (Fig. 4). ABGP186 is the major form in forebrain and cerebellum, but is absent in spinal cord and peripheral nerve. ABGP155 and 140 are present in both brain stem and spinal cord but segregate in forebrain and cerebellum respectively (Fig. 4*a*). ABGP155 is the only detectable form in peripheral nerve. Within the forebrain, ABGP186/155 exhibit a striking segregation between white matter and grey matter, with ABGP186 present almost exclusively in grey matter and ABGP155 in white matter (Fig. 4*b*). The presence of ABGP155 in white matter, and its location in spinal cord and peripheral nerve suggests that this polypeptide is a component of myelinated axon tracts.

NH_2-terminal sequences of ABGP186/155/140 reveal that these polypeptides are related in sequence and are consistent with the possibility that they are alternatively spliced products of the same pre-mRNA (Fig. 4*c*). ABGP155/140 were isolated by adsorption to ankyrin-affinity columns, and separated from ABGP186 by passage through a WGA-affinity column which adsorbed ABGP186 but not ABGP155/140. ABGP155/140 were adsorbed to a Concanavalin A–affinity column, and were subsequently isolated by ion exchange chromatography on a Mono S column (Fig. 4). These proteins were demonstrated to bind to ankyrin by the blot overlay method using radiolabeled ankyrin domain as in Fig. 1 for ABGP186 (not shown). NH_2-terminal sequences of the polypeptides reveals six residues inserted in the sequence of ABGP186 which are missing from ABGP155/140; otherwise the residues determined for all three polypeptides are identical (Fig. 4 *c*).

Measurement of Binding of Ankyrin to Isolated 186-kD and 155/140-kD Polypeptides

Association of ankyrin and glycoproteins isolated from ankyrin-affinity columns was measured in quantitative assays using native proteins immobilized through their carbohydrate residues by adsorption to Concanavalin A–coated beads (Fig. 5). The ankyrin domain (residues 190–947) bound to purified ABGP186 with a K_d of 65 nM and with a stoichiometry close to 1:1. Association of ankyrin domain also was measured with isolated ABGP155/140 that were separated from ABGP186 (Fig. 5). A 270-kD polypeptide identified as the IP3 receptor (data not shown) also was pres-

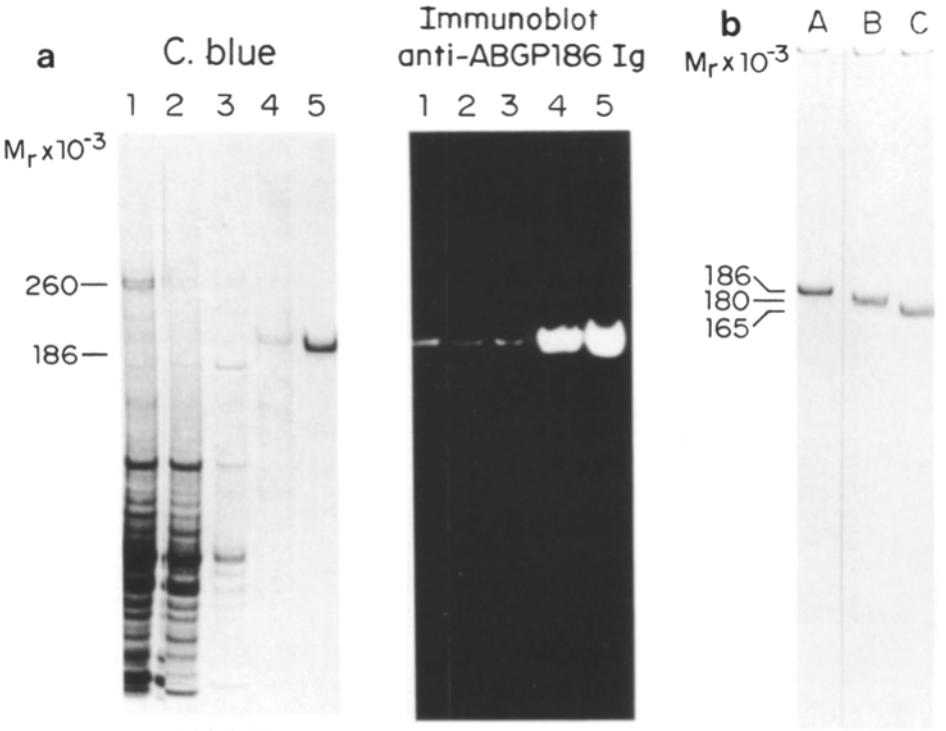


Figure 2. Isolation of the 186-kD ankyrin-binding glycoprotein from adult rat brain. Triton X-100–solubilized proteins from 150 g of adult rat brain were applied to a preparative-scale ankyrin$_B$ (residues 190–947)–agarose affinity column containing 100 mg of immobilized ankyrin$_B$. Adsorbed proteins were eluted (Fig. 1) and applied to a WGA–agarose affinity column (5 ml), and eluted with 0.2 M *N*-acetyl glucosamine (Fig. 1). These proteins were then fractionated by chromatography on a HR5/5 Mono S cation exchange column eluted with a linear gradient of 0–0.5 M NaCl dissolved in column buffer (see Fig. 1). Affinity-purified antibody against the 186-kD polypeptide was isolated from sera of rabbits immunized with the 186-kD polypeptide cut from polyacrylamide gels (see Materials and Methods). Polypeptides were analyzed by SDS–electrophoresis and visualized either by staining with Coomassie blue or by immunoblotting with affinity-purified antibody against the 186-kD ankyrin-binding polypeptide after electrophoretic transfer from SDS-gels to nitrocellulose paper. (Lane *1*) Whole brain membranes; (lane *2*) Triton X-100 extract of brain membranes; (lane *3*) ankyrin-affinity column eluate; (lane *4*) WGA–affinity column eluate; and (lane *5*) peak fraction of the 186-kD polypeptide after chromatography on a Mono S cation exchange column. (*b*) The 186-kD polypeptide was digested for 3 h at 24°C and 14 h at 4°C with endoglycosidases in a 50 μl vol containing 10 μg of polypeptide, 0.2 mU of endo H, or 0.1 U of endo F, and analyzed on a SDS-gel stained with Coomassie blue. (Lane *A*) Undigested polypeptide; (lane *B*) digest with endo H; and (lane *C*) digest with endo F.

ent in this preparation (Fig. 4). However, the 270-kD polypeptide did not interfere with the assay, since it was not adsorbed to the beads under these experimental conditions (data not shown). The affinity of ABGP155/140 for ankyrin was reduced 10-fold compared with ABGP186, with a K_d of 600 nM, although the stoichiometry was ~1:1. The values for affinity and 1:1 molar stoichiometry are consistent with a selective, site-specific interaction between ankyrin and ABGP186, 155, and 140. The differences in affinity between ABGP186 and 155/140 combined with different regional distributions suggests that these polypeptides perform related but distinct functions.

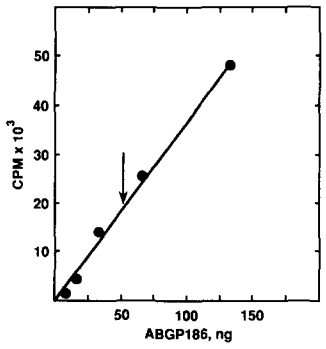


Figure 3. Measurement of levels of the 186-kD ankyrin-binding glycoprotein in adult rat brain membranes. A sample of rat brain membranes and known amounts of the 186-kD ankyrin-binding glycoprotein (Fig. 2) were resolved on SDS gels, the polypeptides electrophoretically transferred to nitrocellulose paper, and an immunoblot performed using affinity-purified Ig against the 186-kD polypeptide and ^{125}I-labeled protein A (see Materials and Methods). Bands corresponding to the 186-kD polypeptide were cut out and assayed for ^{125}I in a gamma counter. Data are plotted as cpm as a function of ng of the 186-kD polypeptide. 18 μg of brain membranes (indicated by *arrow*) contained 52 ng of the 186-kD polypeptide, corresponding to an abundance of 0.3% of the total membrane protein.

Ankyrin-binding Glycoproteins Are Expressed Late in Postnatal Development

Ankyrin-binding glycoproteins are expressed in the forebrain at relatively low levels until after birth, when the level of expression increases over 20-fold between days 10 and 50 (Fig. 6, *left panel*). The 220-kD adult form of ankyrin$_B$ exhibited a similar time course, with expression accelerating after day 10 (Fig. 6). Similar profiles were observed for forebrain and cerebellum (Fig. 6, *right panel*), although ABGP186 is expressed in the cerebellum (<25% of adult values) before day 10. ABGP140 is selectively expressed in the cerebellum, and appears only after day 20, almost 2 wk after ABGP186 and ABGP155. Events known to occur in the cerebellum late in postnatal development after the major phases of neuronal mitosis and migration which coincide with expression of ankyrin-binding glycoproteins include synaptogenesis followed by ensheathing of Purkinje cells by glial cells (Altman, 1972).

Colocalization of Ankyrin-binding Glycoproteins with Ankyrin in Brain and Peripheral Nerve

The localization of ankyrin-binding glycoproteins and ankyrin$_B$ isoforms was determined by immunofluorescence in frozen sections of cerebellum and hippocampus of the forebrain (Fig. 7, *A–D*). In addition, localization of ankyrin-

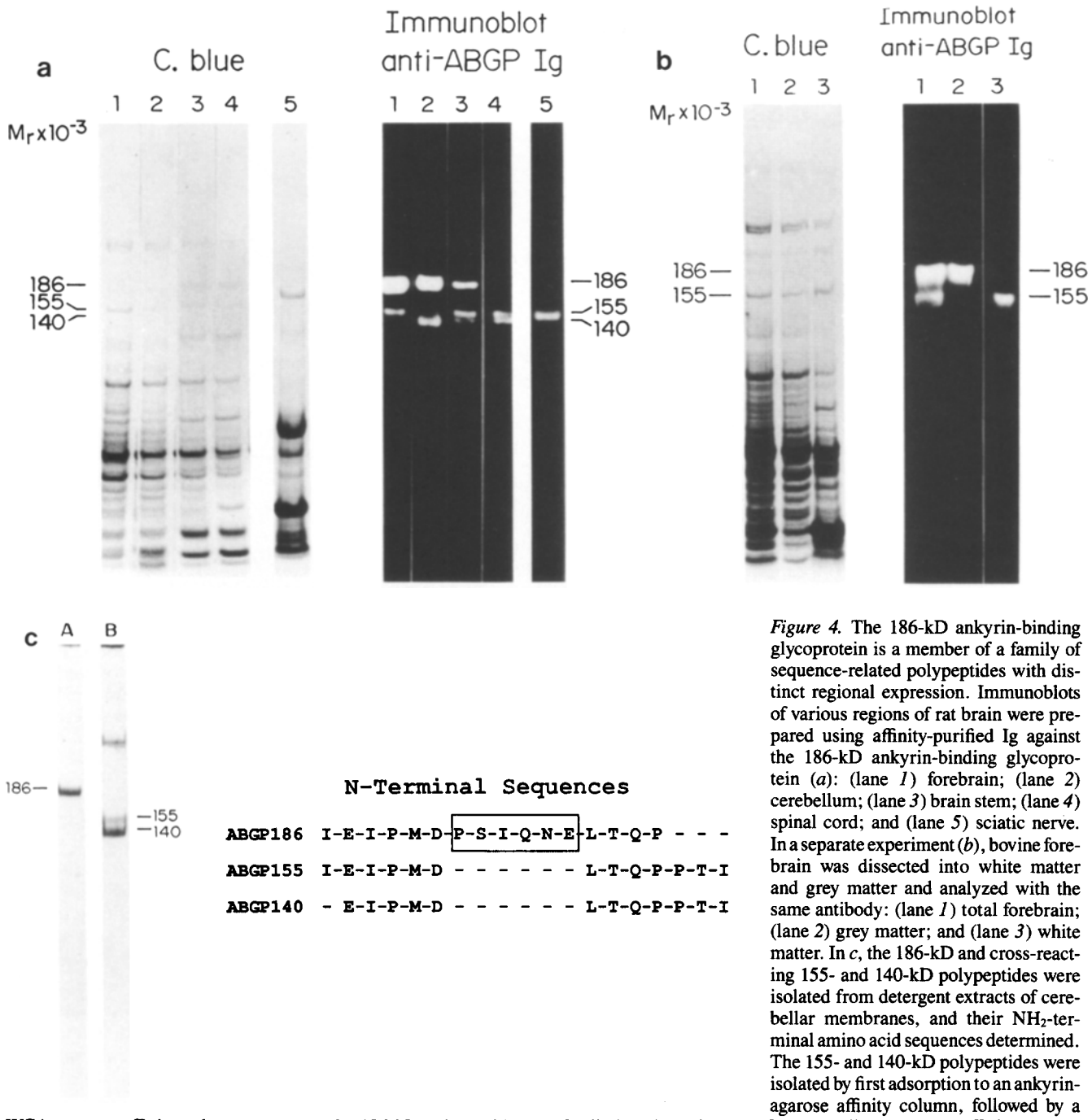


Figure 4. The 186-kD ankyrin-binding glycoprotein is a member of a family of sequence-related polypeptides with distinct regional expression. Immunoblots of various regions of rat brain were prepared using affinity-purified Ig against the 186-kD ankyrin-binding glycoprotein (*a*): (lane *1*) forebrain; (lane *2*) cerebellum; (lane *3*) brain stem; (lane *4*) spinal cord; and (lane *5*) sciatic nerve. In a separate experiment (*b*), bovine forebrain was dissected into white matter and grey matter and analyzed with the same antibody: (lane *1*) total forebrain; (lane *2*) grey matter; and (lane *3*) white matter. In *c*, the 186-kD and cross-reacting 155- and 140-kD polypeptides were isolated from detergent extracts of cerebellar membranes, and their NH_2-terminal amino acid sequences determined. The 155- and 140-kD polypeptides were isolated by first adsorption to an ankyrin-agarose affinity column, followed by a WGA-agarose affinity column to remove the 186-kD polypeptide, and finally by adsorption to a Concanavalin A-agarose affinity column and fractionation on a Mono S cation exchange column (see Fig. 2, and Materials and Methods). NH_2-terminal sequences were determined after electrophoretic transfer to immobilon paper (Matsudaira, 1987).

binding polypeptides was examined in peripheral nerve (Fig. 7 *E*). Ankyrin and the ankyrin-binding polypeptides in the cerebellum are abundant in the molecular layer, which is comprised primarily of dendrites of Purkinje cells and axons of granule cells. Ankyrin and ankyrin-binding proteins also are located on Purkinje cell bodies, and granule cells. The central tracts of myelinated axons exhibit low levels of staining with both antibodies when viewed at low magnification. In the forebrain, ankyrin and ankyrin-binding polypeptides exhibit a general staining consistent with localization in both neurons and glial cells.

Some differences between ankyrin and ankyrin-binding polypeptides in relative staining intensity occur in both cerebellum and forebrain. Ankyrin immunostaining in the cerebellum is more intense in the molecular layer and less intense in granule cells than observed with ankyrin-binding glycoproteins. In the section of forebrain, ankyrin exhibits higher stain intensity in structures that may be mossy fibers, which are bundles of unmyelinated axons. One factor that may contribute to different staining intensity is that Ig against ankyrin reacts with both the major adult form (220 kD) as well as a neonatal (440 kD) alternatively spliced variant of ankyrin

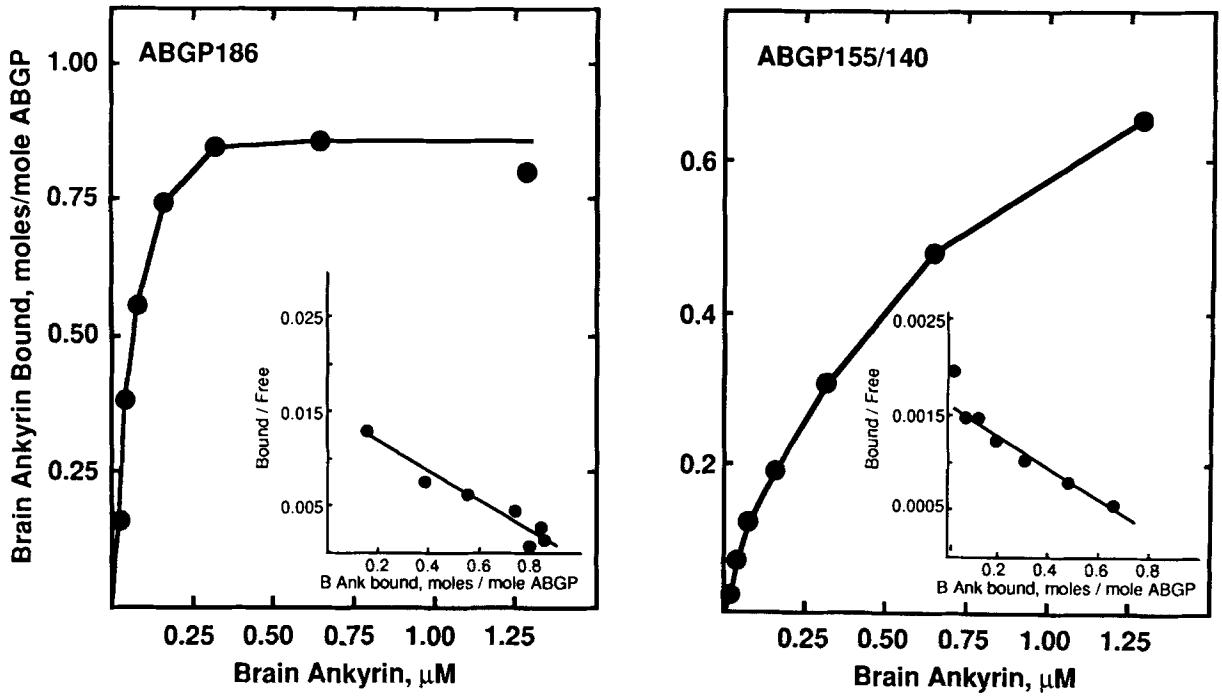


Figure 5. Measurement of association of ^{125}I-labeled ankryin$_B$ (residues 190–947) with ankyrin-binding glycoproteins: 186-kD polypeptide (*left panel*) and 155/140-kD polypeptides (*right panel*). Ankyrin-binding glycoproteins adsorbed to the surface of Concanavalin A-coated beads (25 μl of a 10% suspension) were incubated for 2 h on ice in a 200 μl final volume with increasing concentrations of ^{125}I-labeled ankyrin in assay buffer (Materials and Methods). Samples were layered onto 200 μl of 10% glycerol dissolved in assay buffer, and beads with adsorbed proteins pelleted by centrifugation at 5,000 *g* for 15 min. The tubes were frozen and tips containing the beads cut off and assayed for ^{125}I in a gamma counter. Values for nonspecific binding were determined using a 50-fold excess of unlabeled ankyrin over radiolabeled ankyrin (10 nM), and were subtracted. Data points are the mean of duplicate determinations, and are expressed as moles of ankyrin bound per mole of ankyrin-binding glycoprotein (assuming a monomer), and by the method of Scatchard (*inset*).

(Kunimoto et al., 1991; Otto et al., 1991). The 440-kD ankyrin is highly expressed in the molecular layer and in other regions with unmyelinated axons, and may be associated with distinct membrane protein(s) from the 220-kD ankyrin.

Ankyrin-binding glycoprotein immunoreactivity is highly concentrated at nodes of Ranvier in myelinated axons of peripheral nerve (Fig. 7 *E*). The staining at nodes of Ranvier is likely due to the 155-kD polypeptide which is the only form detectable by immunoblots in peripheral nerve (Fig. 4). Punctate staining could also be detected at higher magnification in myelinated regions of the forebrain and cerebellum, which may represent nodes of Ranvier smaller and more difficult to resolve by light microscopy than those in peripheral nerve (not shown). Since the 155-kD form is the major polypeptide detectable in dissected white matter from forebrain (Fig. 4), the 155-kD polypeptide is likely a component of nodes of Ranvier in the central nervous system as well as peripheral nerve. An isoform of ankyrin distinct from ankyrin$_B$ also is localized at nodes of Ranvier, and is present in unmyelinated axons in peripheral nerves (Kordeli et al., 1990; Kordeli and Bennett, 1991).

Ankyrin-binding Glycoproteins Are Closely Related to Nervous System Cell Adhesion Molecules

An initial cDNA clone was isolated using affinity-purified polyclonal antibody against the 186-kD ankyrin-binding glycoprotein to probe a λgt11 expression library prepared from adult rat brain, and additional cDNAs were subsequently isolated using the first clone as a probe (Fig. 8). A composite sequence encoding 1347 residues was deduced from analysis of multiple overlapping clones, including clones with internal deletions presumably due to alternative splicing of pre-mRNA (Fig. 8). The sequence begins with 24 residues, which presumably represent a transient signal sequence, since they are followed by the NH_2-terminal sequence determined for the 186-kD polypeptide (Fig. 8). Additional matches between predicted and experimentally determined protein sequence occur at residues 155–168 and 708–715. The predicted mol wt for the polypeptide of 145,530 is smaller than the apparent M_r of 165,000 of ABGP186 after digestion with endoglycosidase F (Fig. 2). The difference between predicted and actual migration on SDS-gels could be due to additional sites of glycosylation that were not removed by this digestion, and/or by unusual features of the amino acid sequence resulting in anomalous migration during electrophoresis. Another possibility is that the 186-kD polypeptide includes additional alternatively-spliced segment(s) that were not represented among the cDNA clones isolated.

The predicted sequence of the ankyrin-binding glycoproteins contains five types of domains: (*a*) six Ig domains of the C2 type (residues 25–611); (*b*) four fibronectin type 3 domains (residues 626–1029); (*c*) a 173-residue domain with a high percentage of proline and threonine (residues 1030–1203); (*d*) a hydrophobic stretch of 23 amino acids (residues 1216–1238) representing a putative membrane-

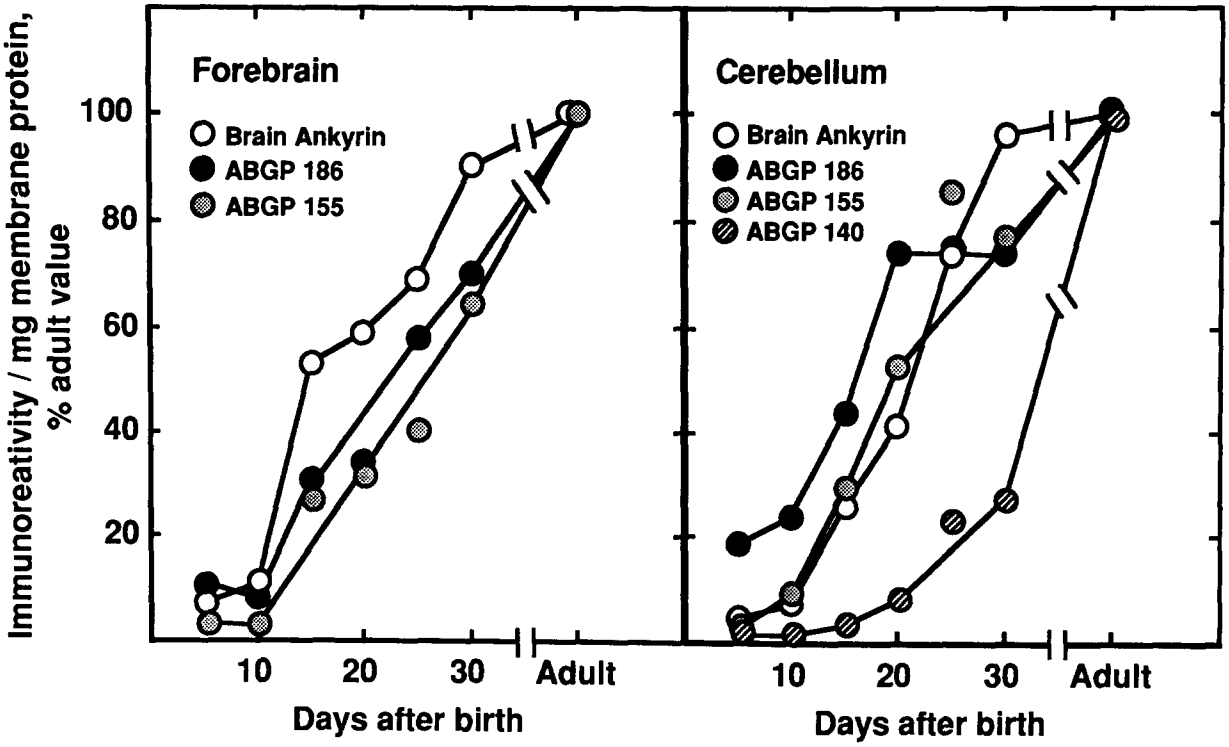


Figure 6. Ankyrin-binding glycoproteins are expressed late in post-natal development of rat brain and parallel expression of the adult form of ankyrin$_B$. Samples of forebrain and cerebellum obtained from rats of various ages were analyzed by immunoblots using ^{125}I-labeled protein A and affinity-purified Ig against the 186-kD ankyrin-binding glycoprotein and ankyrin$_B$ after resolution of polypeptides by SDS-electrophoresis and transfer to nitrocellulose paper (see Materials and Methods). Amounts of immunoreactive 186-, 155-, and 140-kD polypeptides were compared by densitometry of autoradiograms, and these values were then normalized with respect to amount of protein applied to the gel. Protein was estimated by elution of dye from Coomassie blue–stained gels with 25% pyridine followed by measurement of absorbance at 550 nm. The ratios of immunoreactivity/protein are expressed as percent of adult values.

spanning segment; and (*e*) a putative cytoplasmic domain of 109 amino acids (residues 1239–1347). The Ig domains are in the C2 category based on spacing of conserved cysteines 48–55 amino acids apart and distinctive residues near the COOH-terminal cysteines (Williams and Barclay, 1988). The fibronectin type 3 repeats have characteristic conserved tryptophans spaced ~50 amino acids from tyrosines (Patthy, 1990), and the third repeat contains a RGD motif. The 173 residues from 1030–1203 have an unusual amino acid composition with a high proportion of proline (9%), threonine (27%), as well as alanine (9%) and valine (8%). Domains with a similar composition are present in other membrane glycoproteins including the LDL receptor (Yamamoto et al., 1984), platelet glycoprotein Ib (Lopez et al., 1987), and N-CAM (Walsh et al., 1989), and are sites of *O*-glycosylation. Physical studies of mucins indicate that heavily *O*-glycosylated polypeptides are relatively rigid (Jentoft, 1990), suggesting a role for the proline/threonine domain in extending the N-terminal portion of ankyrin-binding glycoprotein above the cell surface.

Four candidate sites for alternative splicing of pre-mRNA can be deduced based on alternative sequences among the cDNA clones: one encoding six residues at the NH_2 terminus (residues 31–36), one encoding 15 residues between Ig and fibronectin type 3 domains (residues 611–625), and two sites in the proline/threonine domain, residues 1030–1035 and 1036–1203 (Fig. 8). The alternate six residues at the NH_2-terminus correspond to the additional amino acids present in the 186-kD polypeptide and are missing from the 155- and 140-kD forms (Fig. 4). This finding provides additional confidence that the cDNA clones correspond to the ankyrin-binding polypeptides, and supports the idea that 186-, 155-, and 140-kD forms result from alternative splicing of the same pre-mRNA. The 15 residues between Ig and fibronectin domains include two prolines and may be configured as an unstructured loop that provides some flexibility between the domains. The potential for multiple alternate sites raises the question of what combination of deletions/insertions are actually expressed as polypeptides. It also follows that the composite sequence does not necessarily correspond to that of any of the major polypeptides.

The amino acid sequence of ankyrin-binding glycoproteins is closely related to previously identified nervous system cell adhesion molecules containing repeated motifs of the Ig type C2 and fibronectin type 3, and highly conserved cytoplasmic domains. The highest degree of similarity with 71% sequence identity is with chicken neurofascin (Fig. 9), with lower degrees of identity of 36 per cent with mouse L1, 47% with chicken Nr-CAM, and 30% with chicken Ng-CAM (Table I). The predicted cytoplasmic domains are the most conserved between ankyrin-binding glycoproteins, neurofascin, L1, Nr-CAM, and Ng-CAM, with some regions that are identical among all five of these proteins (Table I, Fig. 10). *Drosophila neuroglian*, a cell adhesion molecule

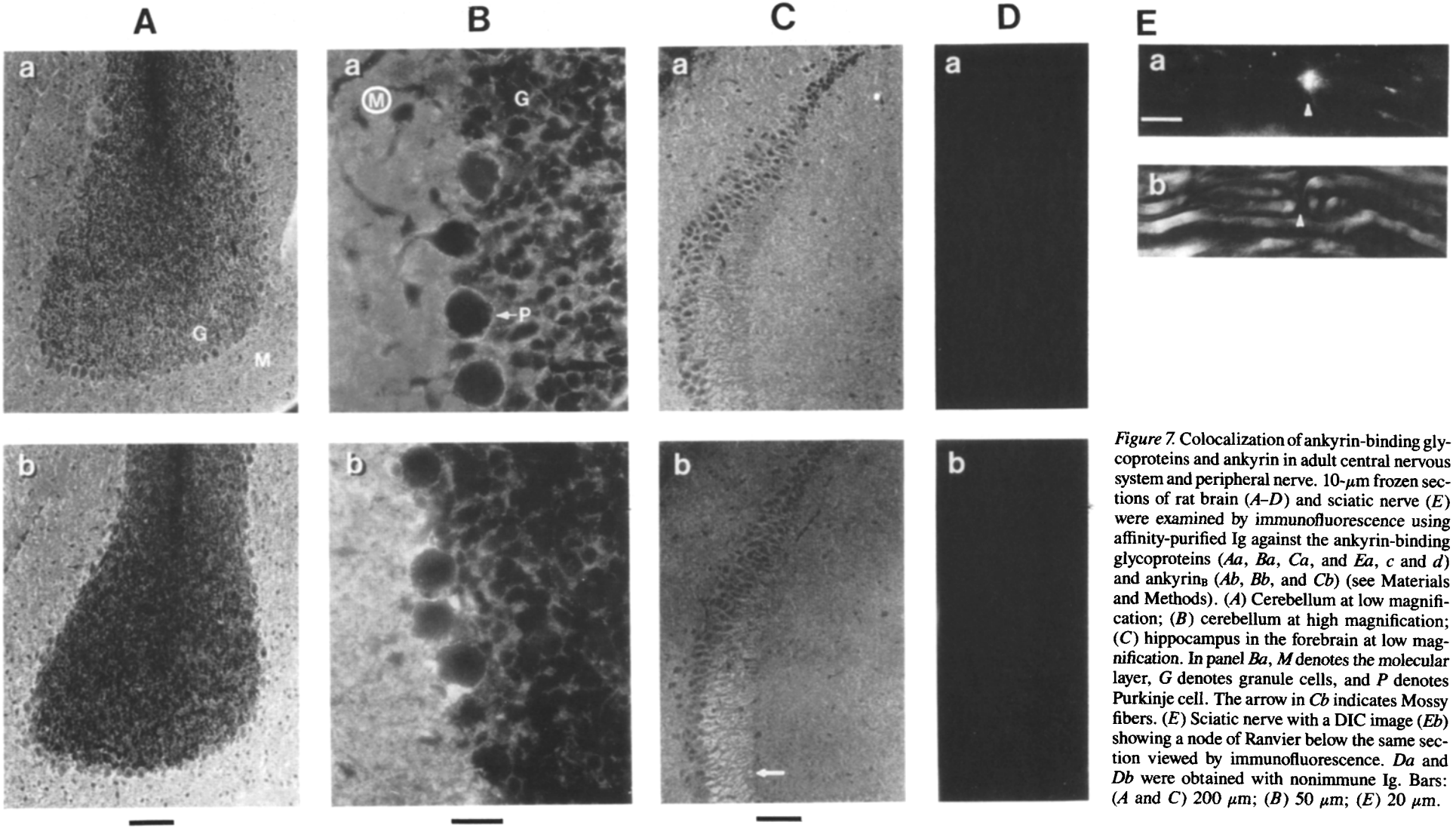


Figure 7. Colocalization of ankyrin-binding glycoproteins and ankyrin in adult central nervous system and peripheral nerve. 10-μm frozen sections of rat brain (*A–D*) and sciatic nerve (*E*) were examined by immunofluorescence using affinity-purified Ig against the ankyrin-binding glycoproteins (*Aa*, *Ba*, *Ca*, and *Ea*, *c* and *d*) and ankyrin$_B$ (*Ab*, *Bb*, and *Cb*) (see Materials and Methods). (*A*) Cerebellum at low magnification; (*B*) cerebellum at high magnification; (*C*) hippocampus in the forebrain at low magnification. In panel *Ba*, *M* denotes the molecular layer, *G* denotes granule cells, and *P* denotes Purkinje cell. The arrow in *Cb* indicates Mossy fibers. (*E*) Sciatic nerve with a DIC image (*Eb*) showing a node of Ranvier below the same section viewed by immunofluorescence. *Da* and *Db* were obtained with nonimmune Ig. Bars: (*A* and *C*) 200 μm; (*B*) 50 μm; (*E*) 20 μm.

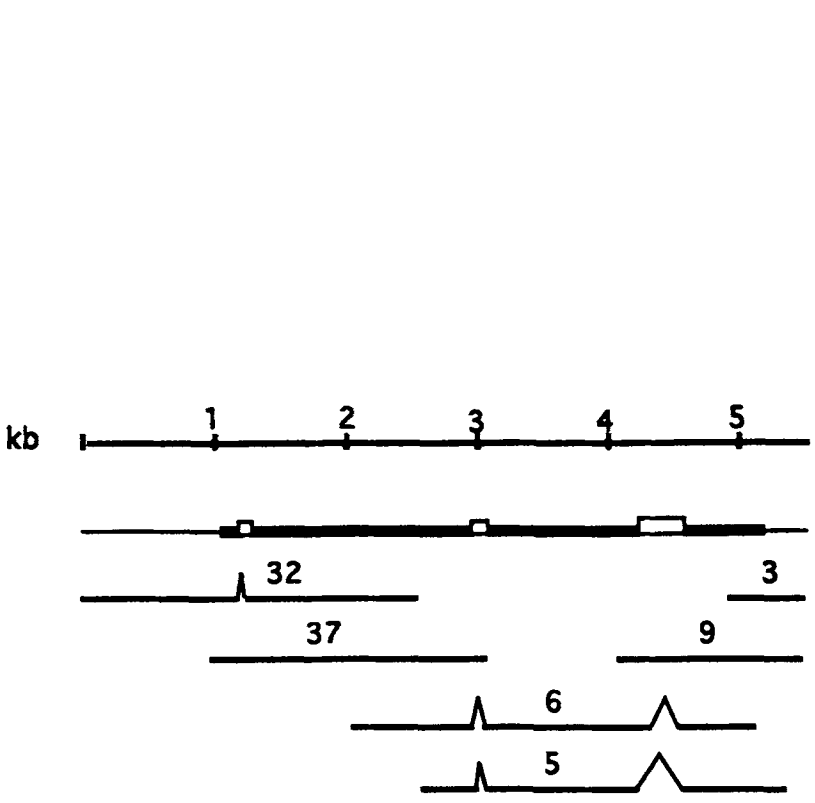


Figure 8. Primary structure of ankyrin-binding glycoproteins deduced from analysis of cDNAs isolated from rat brain. An initial cDNA clone was isolated from a λgt11 expression library prepared from rat brain using poly A and random hexamers as primers (Clonetech) using affinity-purified polyclonal antibody against the 186-kD ankyrin-binding glycoprotein. cDNAs encoding the complete polypeptide were isolated using the first clone and subsequent clones as probes (*left panel*) (see Materials and Methods). A stop codon was present in three independent clones. Portions of sequence corresponding to nucleotides 1130–1142, 2870–2914, 4131–4145, and 4146–4652 were deleted in some of the clones and represent candidates for alternative splicing of pre-mRNA (these are depicted as open segments on the bar). Hydrophobic portions of the sequence representing a predicted signal peptide (residues 1–24) and membrane-spanning region (residues 1216–1238) are underlined and in italics. Alternate sequences are boxed: residues 31–36; 611–625, and 1001–1203. Conserved cysteines of the six Ig-like repeats, and tryptophans and tyrosines of the four fibronectin type 3 repeats are enlarged in bold. Potential *N*-glycosylation sites in the predicted extracellular domain and phosphorylation sites in the cytoplasmic domain are bold and underlined, and an RGD motif (residues 914–916) is in bold. Portions of the deduced sequence confirmed by analysis of NH_2-terminal sequences of polypeptides isolated by Mono Q chromatography of V8 proteolytic digests are underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number L11002.

with similarity to L1 (Bieber et al., 1989) also shares extensive homology with the vertebrate proteins in the cytoplasmic domain (Fig. 10).

Cell adhesion molecules with Ig and fibronectin type 3 domains have the configuration of a relatively rigid rod, based on characterization of N-CAM (Hall and Rutishauser, 1987; Becker et al., 1989) and ICAM (Staunton et al., 1990). The 186-kD ankyrin-binding glycoprotein visualized by EM following rotary shadowing with platinum and carbon also has an elongated shape, 40–60 nm in length (Fig. 11). Some images were twice this length, and could be due to head–head homophilic interactions. Rosettes of 3–5 molecules also were observed, as has been found for N-CAM, and interpreted as association of these proteins through their hydrophobic domains (Becker et al., 1989). The fact that the ankyrin-binding glycoprotein has the predicted configuration in addition to correlation of protein-derived sequence and deduced sequence provides compelling evidence that the cDNA clones actually encode this protein.

Ankyrin-binding glycoproteins and chicken neurofascin share features suggesting that they are encoded by closely related genes and distinguish these proteins from L1, Ng-CAM and Nr-CAM (Fig. 12). The similarity between ankyrin-binding glycoproteins and chicken neurofascin extends throughout the sequence, and includes 80% identity for the 6 Ig domains, 71% for the four fibronectin type 3 domains, 19% for the proline/threonine domain, and 86% for membrane spanning/cytoplasmic domains (Fig. 9; Table I). Both proteins contain four fibronectin type 3 repeats, while the other proteins have five of these repeats. In addition, ankyrin-binding glycoproteins and neurofascin, but not the other proteins, have domains enriched in proline and threonine residues located at the same position between fibronectin type 3 repeats and the predicted membrane-spanning region. Finally, three sites of alternative splicing of pre-mRNA for ankyrin-binding glycoproteins, identified based on isolation of variant cDNA clones (Fig. 8), were also found for neurofascin (Volkmer et al., 1992). The inserted sequences are identical in length in both proteins at the NH_2-terminal site (residues 31–36 for ankyrin-binding glycoprotein). Differences between the proteins are that the insertion between Ig and fibronectin repeats is 12 residues for neurofas-

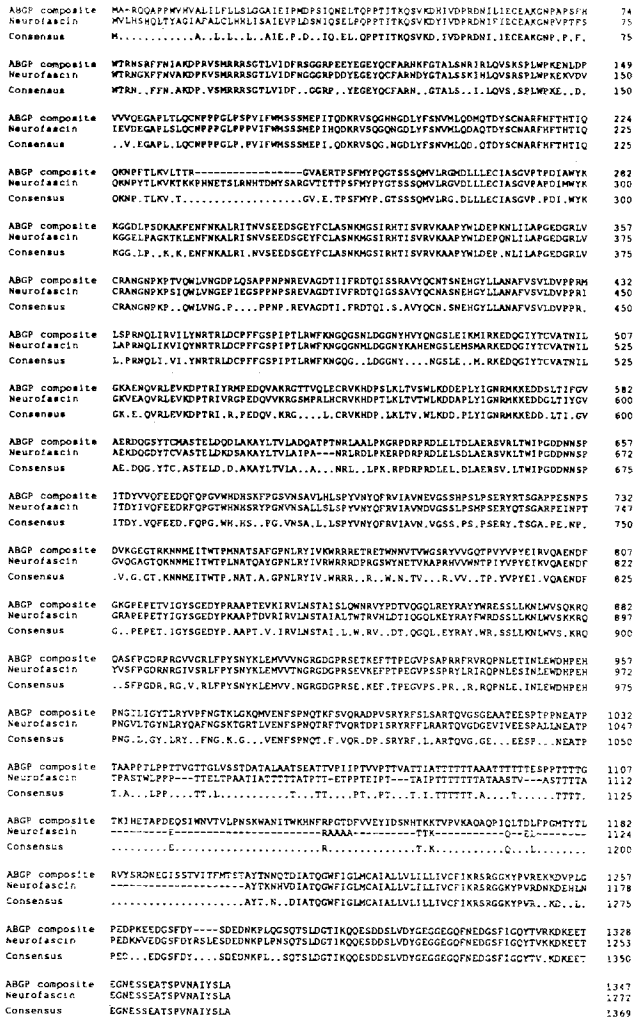


Figure 9. Aligned sequences of rat ankyrin-binding glycoprotein and chicken neurofascin.

cin and 15 for ankyrin-binding glycoproteins, and that the proline/threonine domain of ankyrin-binding glycoproteins is 98 residues longer than that of neurofascin.

The Ankyrin-binding Site Is Located in the COOH-terminal 21 kD (of ABGP186) Containing the Predicted Cytoplasmic Domain

The ankyrin-binding site was localized to the COOH-terminal 21 kD (of ABGP186) based on loss of binding of

Table I. Comparison of Amino Acid Sequence Similarity of ABGP to Vertebrate Nervous System Cell Adhesion Molecules

		Percent identity to ABGP			
	Overall	Domains			
Proteins		Ig	FNIII	PAT	cytoplasmic
Chicken neurofascin	71	80	71	19	86
Chicken Nr-CAM	47	53	45	–	73
Mouse L1	36	38	38	–	50
Chicken Ng-CAM	30	36	29	–	34

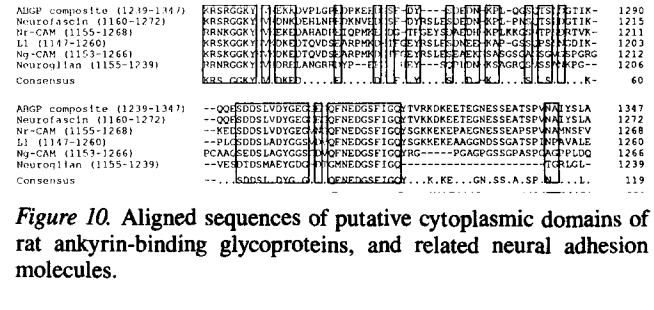


Figure 10. Aligned sequences of putative cytoplasmic domains of rat ankyrin-binding glycoproteins, and related neural adhesion molecules.

^{125}I-labeled ankyrin$_B$ to a 165-kD proteolytic fragment of ABGP186 (Fig. 13). The 165-kD polypeptide was isolated by anion exchange chromatography from a V8 digest of ABGP186. The residues deleted by V8 protease were located at the COOH terminus, since the 165-kD polypeptide retained the same NH_2 terminus as ABGP186 (Fig. 13). The loss of ankyrin-binding activity of the 165-kD polypeptide excludes physiologically irrelevant interactions of ankyrin with the bulk of the extracellular domain. While it is not possible to specify exactly the site of V8 cleavage, the 165-kD polypeptide is almost certainly missing the COOH terminal 108 residues comprising the cytoplasmic domain.

Discussion

This report provides the molecular basis for a significant class of membrane–cytoskeletal interactions in the adult mammalian nervous system involving a membrane-spanning nervous system cell adhesion molecule, and ankyrin, a structural protein located on the cytoplasmic surface of the plasma membrane. The ankyrin-binding membrane glycoproteins (ABGPs) discovered in this study include several alternatively spliced variants with 72% sequence identity with chicken neurofascin, a recently described member of a subclass of nervous system cell adhesion molecules with conserved cytoplasmic domains (Volkmer et al., 1992). ABGP polypeptides have the following features consistent with a role as ankyrin-binding proteins in vitro and in vivo: (*a*) ABGPs and ankyrin associate as pure proteins in a 1:1 molar stoichiometry (Fig. 5); (*b*) the ankyrin-binding site is located in the COOH-terminal 21 kD (of ABGP186) which contains the predicted cytoplasmic domain (Fig. 13); (*c*) ABGP186 is expressed at approximately the same levels as ankyrin (15 pmoles/milligram of membrane protein) (Fig. 3); (*d*) ABGP polypeptides are co-expressed with the adult form of ankyrin$_B$ late in postnatal development and are colocalized with ankyrin$_B$ by immunofluorescence (Figs. 6 and 7). Associations of ABGPs with ankyrin, of ankyrin with spectrin (Davis and Bennett, 1984), and of spectrin with actin (Bennett et al., 1982; Glenney et al., 1982) provide an example of a series of protein–protein interactions extending from the extracellular space to the cytoplasm that have been defined in terms of affinity and stoichiometry with pure components. Other candidates for membrane–cytoskeletal interactions in brain and other tissues that have yet to be characterized at this level presently include association of N-CAM180 with spectrin (Pollerberg et al., 1987), of cadherins and catenins with actin (Takeichi, 1991), and of E-cadherin with spectrin and ankyrin (Nelson et al., 1990).

Neurofascin was initially characterized as a membrane protein hypothesized to play a role in extension of neurites

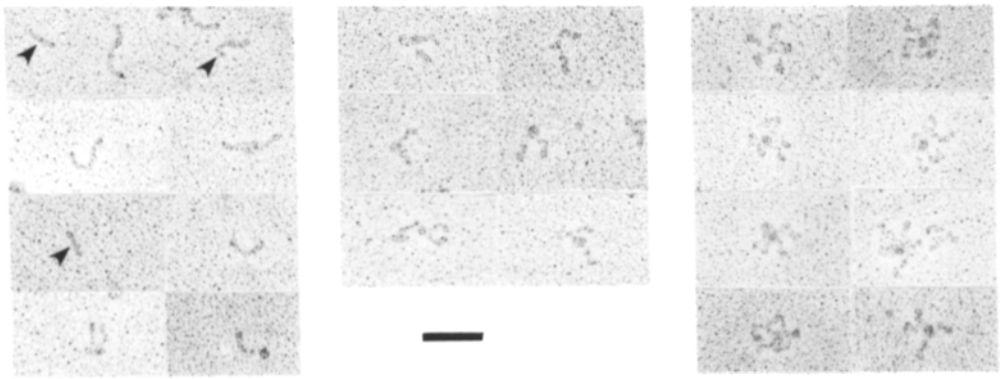


Figure 11. Visualization of the 186-kD ankyrin-binding glycoprotein by electron microscopy. The 186-kD ankyrin-binding glycoprotein (300 μg/ml) in a buffer containing 0.1% Triton X-100, 0.01% phosphatidylcholine, 10 mM Hepes, pH 7.4, was diluted 20-fold with 30% glycerol, 0.1 M ammonium formate, pH 7.0 and immediately sprayed onto freshly cleaved mica. The samples were then rotary shadowed at an angle of six degrees with platinum followed by carbon, and the replicas visualized by EM (see Materials and Methods). Bar, 100 nm.

and stabilization of bundles of axons during development of the nervous system (Rathjen et al., 1987; Volkmer et al., 1992). A significant discovery of this study is that ABGPs, which are closely related to neurofascin, are most prominent in adult brain, with a 10–20-fold increased expression after the major phases of neuronal migration (Fig. 6). It is not certain at this time whether the ABGP polypeptides are encoded by the identical gene as chicken neurofascin. The similarity in amino acid sequence and conservation of sites of alternative splicing strongly support the interpretation that ABGPs and neurofascin genes share a common ancestor. However, the major differences in developmental expression and divergence in some areas of the sequence (Fig. 9) suggest that ABGPs and neurofascin represent products of gene duplication events that have subsequently evolved in parallel with distinct roles in development. Important issues for the future will be to determine if indeed multiple copies of the ABGP and neurofascin genes exist, or if the same gene plays roles both in fetal development and in adult brain. It also is conceivable that rats and chickens simply differ in usage of neurofascin.

The high expression of rat ABGP in postnatal and adult brain strongly suggests the function(s) of this protein includes activities in addition to those in prenatal development. A prominent role of ABGPs in adult mammalian brain has potential clinical implications, since defects in these proteins may be compatible with survival but with impaired neurological or mental functions. It is of interest in this regard that abnormal splicing of human L1 premRNA, a related cell adhesion molecule, results in hydrocephalus and mental retardation (Rosenthal et al., 1992). Another potential clinical implication is that ABGPs may play a role as receptors for neurotropic viruses as occurs for Rhinoviruses in lymphocytes (Staunton et al., 1990).

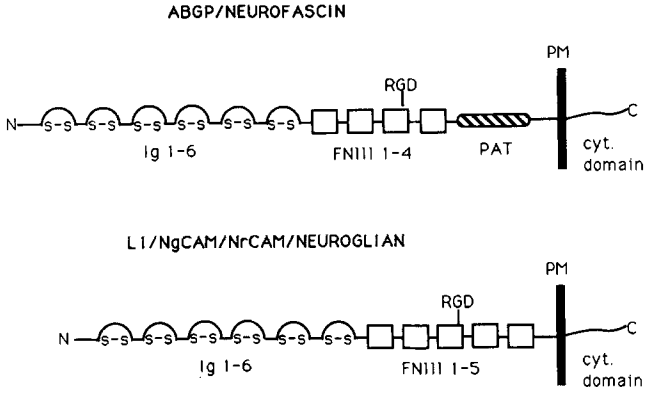
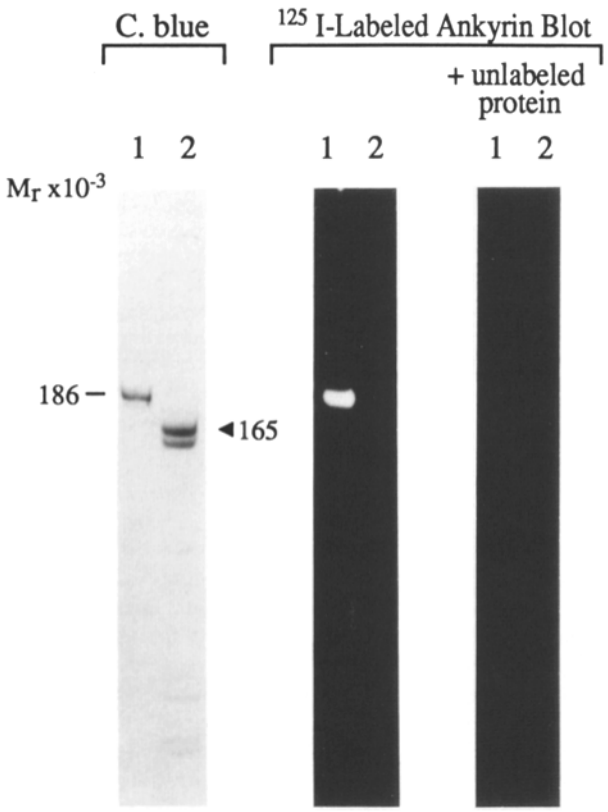


Figure 12. Schematic model for the domain organization of ankyrin-binding glycoproteins, neurofascin and related nervous system cell adhesion molecules.

Predicted cytoplasmic domains of ABGPs, neurofascin, and related proteins L1, Ng-CAM, Nr–CAM, and neuroglian are the most conserved domain of this group of cell adhesion molecules. In addition to sequence similarity, cytoplasmic domains of chicken neurofascin (Volkmer et al., 1992) rat and human L1 (Miura et al., 1991; Kobayashi et al., 1991; Reid and Hemperly, 1992) and chicken Nr-CAM (Kayyem et al., 1992) also share alternative splicing involving a tetrapeptide, RSLE. The cytoplasmic domain of rat ABGP is predicted to contain the ankyrin-binding site (Fig. 13), suggesting the possibility that other members of this group also interact with either the same ankyrin or a related member of the ankyrin family. A 440-kD alternatively spliced form of ankyrin$_B$ is expressed before birth (Kunimoto et al., 1991), and it also is possible that other forms of ankyrin are present in early development. Association of this family of nervous system cell adhesion molecules with ankyrins could play a role in the polarized distribution of many of these proteins to axons and other specialized cell domains (Rathjen and Jessels, 1991).

Nervous system cell adhesion molecules and ankyrins are families of proteins that are each capable of multiple types of interactions. Association between the two families provides the potential for considerable diversity in molecular connections between the extracellular and intracellular compartments. ABGPs include several polypeptides derived from alternative splicing of pre-mRNA, and these forms exhibit differences in affinity for ankyrin, regional distribution, and time of expression during development (Fig. 4). In addition to alternative splicing, another level of diversity is provided by the subgroup of cell adhesion molecules with cytoplasmic domains related to ABGP and neurofascin which are candidates to associate with ankyrins (Fig. 11). The cyto-



N-Terminal Sequence

ABGP186 I-E-I-P-M-D-P-S-I-Q-N-E-L-T-Q-P-
V8 165 I-E-I-P-M-D-P-S-I-Q-N-E-L-T-Q-P-

Figure 13. Localization of the ankyrin-binding site to the COOH-terminal 21-kD polypeptide of ABGP186. ABGP186 (0.1 mg/ml in a buffer containing 50 mM NaCl, 10 mM Hepes, pH 7.4, 0.5% Triton X-100, 0.01% phosphatidylcholine, 0.5 mM DTT, 1 mM Na azide) was digested with staphylococcal V8 protease (20 μg/ml for 60 min at 24°C), and the products resolved by chromatography on a Mono Q anion exchange column. A 165-kD polypeptide was resolved which retained the NH_2-terminus based on amino acid sequence, and was missing the COOH-terminal 21-kD polypeptide. Binding of ^{125}I-labeled ankyrin$_B$ (residues 190–947) to 186- and 165-kD polypeptides was determined after electrophoretic transfer of polypeptides to nitrocellulose paper as in Fig. 1. (*Left panel*) Coomassie blue–stained polypeptides; (*right panels*) autoradiograms of nitrocellulose paper incubated with 36 nM ^{125}I-labeled ankyrin$_B$ (residues 190–947), either alone or with a 75-fold excess unlabeled ankyrin to displace nonspecific binding.

plasmic domains of these proteins contain several potential phosphorylation sites which are used in the case of L1 (Sadoul et al., 1989), and could provide a mechanism for dynamic regulation of association with ankyrin. Interactions in addition to homophilic associations have been observed for other cell adhesion molecules containing Ig and Fibronectin type 3 repeats and may also be available to ABGPs. Examples include binding to soluble extracellular matrix molecules (Reyes et al., 1990; Kuhn et al., 1991; Rathjen et al., 1991), lateral association with other cell adhesion molecules to form complexes capable of cell–cell interactions (Kadmon et al., 1990), and heterophilic interactions with integral membrane proteins on adjacent cells (Marlin and Springer, 1987). It is clear that a simple interaction between ankyrins and ABGPs and perhaps other cell adhesion molecules with conserved cytoplasmic domains can, in principle, be used in a variety of specialized contexts in the nervous system. One example of such a specialized membrane domain is the node of Ranvier, where a 155-kD form of ABGP is localized (Fig. 7), and which also contains an isoform of ankyrin (Kordeli et al., 1990; Kordeli and Bennett, 1991).

A future challenge will be to determine the functional importance of ankyrin–ABGP interactions. Possibilities include a role in developing and/or maintaining specialized membrane domains such as unmyelinated axons and the node of Ranvier discussed above. Another consequence of associations between these proteins may be structural support for the adult brain, which is dependent on cell–cell contacts and lacks a collagen-based basement membrane used by cells in most tissues. Coupling between the extracellular domain of ABGP and the cytoskeleton would provide a mechanical buffer allowing distribution of shear stresses and deformations throughout the tissue. It is of interest in this regard that elongated molecules with similar dimensions to ABGP (Fig. 11) have been observed extending between the extracellular surfaces of neurons and glial cells at nodes of Ranvier (Ichimura and Ellisman, 1991). The situation in brain tissue thus may be formally analogous to the epidermis, which has layers of keratinocytes attached to each other by desmosomes on their outer surfaces which are interconnected by intermediate filaments in the cytoplasm.

In summary, this study provides evidence for interactions between ankyrin on the cytoplasmic surface of the plasma membrane and a family of alternatively spliced membrane-spanning nervous system cell adhesion molecules closely related to neurofascin which are highly expressed in adult brain and are candidates to comprise a major portion of ankyrin-binding sites in brain membranes. A physiological consequence of convergence of cytoskeletal, transmembrane, and intercellular connections in adult brain may be stabilization of the structure of the nervous system so that this intricate arrangement of cells can survive the traumas of everyday life.

Brenda Sampson is gratefully acknowledged for help in preparing the manuscript. Drs. D. Gilligan, E. Kordeli, and S. Lambert are thanked for comments and suggestions, and for help in editing the manuscript.

Received for publication 10 November 1992 and in revised form 21 December 1992.

References

Altman, J. 1972. Postnatal development of the cerebellar cortex in the rat. *J. Comp. Neurol.* 145:399–464.

Becker, J., H. Erickson, S. Hoffman, B. Cunningham, and G. Edelman. 1989. Topology of cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 86: 1088–1092.

Bennett, V. 1979. Immunoreactive forms of human erythrocyte ankyrin are present in diverse cells and tissues. *Nature (Lond.).* 281:597–599.

Bennett, V. 1990. The spectrin-based membrane skeleton: a multipotential adaptor between membrane and cytoplasm. *Physiol. Rev.* 70:1029–1066.

Bennett, V. 1992. Ankyrins: adaptors between diverse plasma membrane proteins and cytoplasm. *J. Biol. Chem.* 267:8703–8706.

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

Bieber, A. J., P. M. Snow, M. Hortsch, N. H. Patel, J. R. Jacobs, Z. R. Traquina, J. Schilling, and C. S. Goodman. 1989. Drosophila neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural cell adhesion molecule L1. *Cell.* 59:447–460.

Burgoon, M. P., M. Grumet, V. Mauro, G. M. Edelman, and B. A. Cunningham. 1991. Structure of the chicken neuron-glia cell adhesion molecule, Ng-CAM: origin of the polypeptides and relation to the Ig superfamily. *J. Cell Biol.* 112:1017–1029.

Davis, J., and V. Bennett. 1984. Brain ankyrin—a membrane-associated protein with binding sites for spectrin, tubulin and the cytoplasmic domain of the erythrocyte anion channel. *J. Biol. Chem.* 259:13550–13559.

Davis, J., and V. Bennett. 1986. Association of brain ankyrin with brain membranes and isolation of active proteolytic fragments of membrane-associated ankyrin-binding proteins. *J. Biol. Chem.* 261:16198–16206.

Davis, L., and V. Bennett. 1990. Mapping the binding sites of human erythrocyte ankyrin of the anion exchanger and spectrin. *J. Biol. Chem.* 265: 10589–10596.

Davis, L., E. Otto, and V. Bennett. 1991. Specific 33-residue repeat(s) of erythrocyte ankyrin associate with the anion exchanger. *J. Biol. Chem.* 266: 11163–11169.

Glenney, J., P. Glenney, M. Osborn, and K. Weber. 1982. An F-actin- and calmodulin-binding protein from isolated intestinal brush borders has a morphology related to spectrin. *Cell.* 28:843–854.

Grumet, M., V. Mauro, M. P. Burgoon, G. M. Edelman, and B. A. Cunningham. 1991. Structure of a new nervous system glycoprotein, Nr-CAM and its relationship to subgroups of neural cell adhesion molecules. *J. Cell Biol.* 113:1399–1412.

Hall, A. K., and U. Rutishauser. 1987. Visualization of neural cell adhesion molecule by electron microscopy. *J. Cell Biol.* 104:1579–1586.

Ibraghimov-Beskrovnaya, O., J. M. Ervasti, C. J. Leveille, C. A. Slaughter, S. W. Sernett, and K. Campbell. 1992. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature (Lond.).* 355:690–702.

Ichimura, T., and M. H. Ellisman. 1991. Three-dimensional fine structure of cytoskeletal-membrane interactions at nodes of Ranvier. *J. Neurocytol.* 20:667–681.

Jentoft, N. 1990. Why are proteins O-glycosylated? *Trends Biochem. Sci.* 15:291–293.

Kadmon, G., A. Kowitz, P. Alevogt, and M. Schachner. 1990. The neural cell adhesion molecule N-CAM enhances L1-dependent cell-cell interactions. *J. Cell Biol.* 110:193–208.

Kalomiris, E. L., and L. Y. Bourguignon. 1988. Mouse T lymphoma cells contain a transmembrane glycoprotein (GP85) that binds ankyrin. *J. Cell Biol.* 106:319–327.

Kayyem, J. F., J. M. Roman, E. de la Rosa, U. Schwarz and W. J. Dreyer. 1992. Bravo/Nr-CAM is closely related to the cell adhesion molecules L1 and Ng-CAM and has a similar heterodimer structure. *J. Cell Biol.* 118:1259–1270.

Kobayashi, M., M. Miura, H. Asou, and K. Uyemura. 1991. Molecular cloning of cell adhesion molecule L1 from human nervous tissue: a comparison of the primary sequences of L1 molecules of different origin. *Biochim. Biophys. Acta.* 1090:238–240.

Koob, R., M. Zimmermann, W. Schone, and D. Drenckhahn. 1987. Colocalization and coprecipitation of ankyrin and Na^+,K^+-ATPase in kidney epithelial cells. *Eur. J. Cell Biol.* 45:230–237.

Kordeli, E., and V. Bennett. 1991. Distinct ankyrin isoforms at neuron cell bodies and nodes of Ranvier resolved using erythrocyte ankyrin-deficient mice. *J. Cell Biol.* 114:1243–1259.

Kordeli, E., J. Davis, B. Trapp, and V. Bennett. 1990. An isoform of ankyrin is localized at nodes of Ranvier in myelinated axons of central and peripheral nerves. *J. Cell Biol.* 110:1341–1352.

Kuhn, T. B., E. T. Stoeckli, M. A. Condrau, F. G. Rathjen, and P. Sonderegger. 1991. Neurite outgrowth on immobilized axonin-1 is mediated by heterophilic interaction with L1. *J. Cell Biol.* 115:1113–1126.

Kunimoto, M., E. Otto, and V. Bennett. 1991. A new 440-kD isoform is the major ankyrin in neonatal rat brain. *J. Cell Biol.* 115:1319–1331.

Lopez, J., D. Chung, K. Fujikawa, F. S. Hagen, T. Papayannopoulou, and G. Roth. 1987. Cloning of the a chain of human platelet glycoprotein 1b: a transmembrane protein with homology to leucine-rich a_2 glycoprotein. *Proc. Natl. Acad. Sci. USA.* 84:5615–5619.

Marlin, S. D., and T. A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated Antigen 1 (LFA-1). *Cell.* 51:813–819.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262: 10035–10038.

Michaely, P., and V. Bennett. 1992. The ANK repeat: a ubiquitous motif involved in macromolecular recognition. *Trends Cell Biol.* 2:127–129.

Miura, M., M. Kobayashi, H. Asou, and K. Uyemura. 1991. Molecular cloning of cDNA encoding the rat neural adhesion molecule L1. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 289:91–95.

Moos, M., R. Tacke, H. Scherer, D. Teplon, K. Fruh, and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature (Lond.).* 334:701–703.

Morrow, J. S., C. D. Cianci, T. Ardito, A. S. Mann, and M. Kashgarian. 1989. Ankyrin links fodrin to the alpha subunit of Na,K-ATPase in Madin-Darby canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* 108:455–465.

Nelson, W. J., and P. J. Veshnock. 1987. Ankyrin binding to the (Na^+ + K^+) ATPase and implications for the organization of membrane domains in polarized cells. *Nature (Lond.).* 328:533–535.

Nelson, W. J., E. Shore, A. Wang, and R. Hammerton. 1990. Identification of a membrane-cytoskeletal complex containing the cell adhesion molecule (E-cadherin), ankyrin, and fodrin in Madin-Darby Canine Kidney epithelial cells. *J. Cell Biol.* 110:349–357.

Otto, E., M. Kunimoto, T. McLaughlin, and V. Bennett. 1991. Isolation and characterization of cDNAs encoding human brain ankyrins reveal a family of alternatively spliced genes. *J. Cell Biol.* 114:241–253.

Patthy, L. 1990. Homology of a domain of the growth hormone/prolactin receptor with type III modules of fibronectin. *Cell.* 61:13–14.

Rathjen, F. G., and T. M. Jessel. 1991. Glycoproteins that regulate the growth and guidance of vertebrate axons: domains and dynamics of the immunoglobulin/fibronectin type III subfamily. *The Neurosciences.* 3:297–307.

Rathjen, F. G., J. M. Wolff, S. Chang, F. Bonhoeffer, and J. Raper. 1987. Neurofascin: a novel chick cell-surface glycoprotein involved in neurite-neurite interactions. *Cell.* 51:841–849.

Rathjen, F. G., J. M. Wolff, and R. Chiquet-Ehrismann. 1991. Restrictin: a chick neural extracellular matrix protein involved in cell attachment copurifies with the cell recognition molecule F11. *Development (Camb.).* 113:151–164.

Reid, R. A. and J. J. Hemperly. 1992. Variants of human L1 cell adhesion-molecule arise through alternate splicing of RNA. *J. Mol. Neurosci.* 3: 127–135.

Reyes, A. A., R. Akeson, L. Brezina, and G. J. Cole. 1990. Structural requirements for neural cell adhesion molecule-heparin interaction. *Cell Regul.* 1:567–576.

Rosenthal, A., M. Jonet, and S. Kenwrich. 1992. Aberrant splicing of neural cell adhesion molecule L1 mRNA in a family with x-linked hydrocephalus. *Nat. Genet.* 2:107–112.

Sadoul, R., F. Kirchhoff, and M. Schachner. 1989. A protein kinase activity is associated with and specifically phosphorylates the neural cell adhesion molecule L1. *J. Neurochem.* 53:1471–1478.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Srinivasan, Y., L. Elmer, J. Davis, V. Bennett, and K. Angelides. 1988. Ankyrin and spectrin associate with voltage-dependent sodium channels in brain. *Nature (Lond.).* 333:177–180.

Srinivasan, Y., M. Lewallen, and K. J. Angelides. 1992. Mapping the binding site on ankyrin for the voltage-dependent sodium channel from brain. *J. Biol. Chem.* 267:7483–7489.

Staunton, D. E., M. L. Dustin, H. P. Erickson, and T. A. Springer. 1990. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and Rhinovirus. *Cell.* 61:243–254.

Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorf. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60–89.

Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251:1451–1455.

Treharne, K. J., D. Rayner, and A. J. Bains. 1988. Identification and partial purification of ABGP205, an integral membrane glycoprotein from brain that binds ankyrin. *Biochem. J.* 253:345–350.

Volkmer, H., B. Hassel, J. M. Wolff, R. Frank, and F. G. Rathjen. 1992. Structure of the axonal surface recognition molecule neurofascin and its relationship to a neural subgroup of the immunoglobulin superfamily. *J. Cell Biol.* 118:149–161.

Walsh, F. S., R. B. Parekh, S. E. Moore, G. Dickson, C. H. Barton, H. Gower, R. A. Dwek, and T. W. Rodemarcher. 1989. Tissue-specific O-linked glycosylation of the neural cell adhesion molecule (N-CAM). *Development (Camb.).* 105:803–811.

Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily—Domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381–405.

Yamamoto, T., C. G. Davis, M. Brown, W. Schneider, M. Casey, J. Goldstein, and D. Russell. 1984. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell.* 39:27–38.