A Neuron-specific Isoform of Brain Ankyrin, 440-kD Ankyrin$_B$, Is Targeted to the Axons of Rat Cerebellar Neurons

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Abstract. Two isoforms of brain ankyrin, 440- and 220-kD ankyrin$_B$, are generated from the same gene by alternative splicing of pre-mRNA. The larger isoform shares the same NH_2-terminal and COOH-terminal domains to the smaller isoform and contains, in addition, a unique inserted domain of about 220-kD in size (Kunimoto, M., E. Otto, and V. Bennett. 1991. *J. Cell Biol.* 115:1319–1331). Both Isoforms were expressed in primary cerebellar cells in a manner similar to that in vivo; the larger isoform appeared first when axogenesis is actively conducted and the smaller isoform came up later. 440-kD ankyrin$_B$ was localized in the axons of cerebellar neurons both in vivo and in vitro using an antibody raised against the insert region, while 220-kD isoform was rather localized in the cell bodies and dendrites of neurons by a specific antibody prepared using a synthetic peptide corresponding to the splice site as antigen. Astroglia cells also expressed 220-kD ankyrin$_B$ but not the 440-kD isoform. These results indicate that 440-kD ankyrin$_B$ is a neuron-specific isoform targeted to the axons and its unique inserted domain is essential for the targeting.

ANKYRINS are a family of spectrin-binding proteins that link the spectrin/actin network to the cytoplasmic domain of integral proteins that include ion channels and cell adhesion molecules (Bennett and Gilligan, 1993; Davis et al., 1993). Ankyrins contain three domains; an NH_2-terminal 89–95-kD membrane binding domain (Davis and Bennett, 1990), a 62-kD domain that binds spectrin (Bennett, 1978) and a COOH-terminal domain that is the target of alternative splicing and is the most variable domain among different ankyrins. The membrane binding domain contains 24 tandem repeats of 33-amino acids which are necessary and sufficient for association of ankyrin with the erythrocyte anion exchanger (Davis and Bennett, 1990; Davis et al., 1991), the voltage-dependent sodium channel (Srinivasan et al., 1992) and nervous system cell adhesion molecules related to L1 and neurofascin (Davis et al., 1993; Davis and Bennett, 1994).

Three different ankyrins are currently known to be expressed in brain tissue: ankyrin$_R$, which is also expressed in erythrocytes; ankyrin$_B$, which is the major ankyrin in the brain; and ankyrin$_G$, which is localized in axonal initial segments and nodes of Ranvier of myelinated axons (Kordeli et al., 1990, 1995). Ankyrin$_B$ includes two isoforms of 220-kD and 440-kD which are products of alternatively spliced pre-mRNAs encoded by a single gene (Kunimoto et al., 1991; Otto et al., 1991). 220-kD ankyrin$_B$ is the major isoform in adult brain (Kordeli et al., 1990), while 440-kD ankyrin$_B$ is maximally expressed in developing neonatal rat brain, with a peak at postnatal day 10, and decreases to about 30% of the maximal level in adult brain (Kunimoto et al., 1991). 440-kD ankyrin$_B$ shares the same NH_2-terminal and COOH-terminal domains as 220-kD ankyrin$_B$ and contains, in addition, an inserted domain of 220-kD located between the membrane/spectrin-binding domains and the COOH-terminal domain (Kunimoto et al., 1991; Otto et al., 1991; Chan et al., 1993). Immunocytochemical staining of developing rat cerebellum (Kunimoto et al., 1991) and optic nerve (Chan et al., 1993) implies that 440-kD ankyrin$_B$ is localized to unmyelinated axons and premyelinated axons.

Localization of 220-kD ankyrin$_B$, however, has not been clearly demonstrated, because this isoform is totally subsumed within the 440-kD isoform, which prevents the production of specific antibodies by standard strategies using recombinant proteins as antigens.

In this paper, a synthetic peptide corresponding to the splice site was designed to prepare a polyclonal antibody which can recognize the 220-kD isoform specifically. Using these antibodies the expression and localization of the two ankyrin$_B$ isoforms in cerebellar cells in vivo and in vitro was investigated, and the evidence showing that 440-kD ankyrin$_B$ is a neuron-specific isoform and the inserted domain of 440-kD ankyrin$_B$ is essential for the targeting of this isoform to the axon is presented.

Materials and Methods

Materials

^{125}I-Protein A was from ICN Radiochemicals (Irvine, CA). All tissue cul-

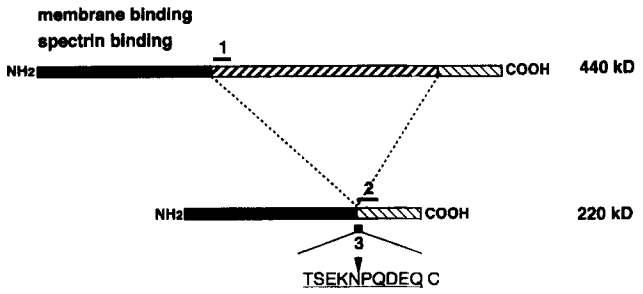


Figure 1. Schematic organization of ankyrin$_B$ isoforms. Recombinant proteins corresponding to regions 1 and 2 were used as antigens to prepare polyclonal antibodies 1 and 2, respectively and synthetic peptide corresponding to the region 3 was used to prepare antibody 3.

ture media, sera and supplements were from GIBCO BRL (Gaithersburg, MD). Mouse monoclonal antibodies against microtubule associated protein 2 (MAP2),[1] glial fibrillary acidic protein (GFAP), growth-associated protein-43 (GAP-43), and tau were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Rabbit anti-mouse IgG, goat anti-rabbit IgG (TRITC-labeled), goat anti-mouse IgG (FITC-labeled), normal goat serum, Triton-X-100, pepstatin A, leupeptin, and diisopropyl fluorophosphate (DFP) were from Sigma Chemical Co. (St. Louis, MO). Peptides were synthesized with a peptide synthesizer (model 9050; MilliGen/Biosearch).

Antibodies Preparation

Antibodies 1 and 2, both rabbit polyclonal, were prepared using recombinant proteins as antigens as described before (Kunimoto et al., 1991). Antibody 1 raised against the recombinant protein corresponding to region 1 in Fig. 1 recognizes 440-kD isoform specifically. Antibody 2 raised against region 2 in Fig. 1 recognizes both 440- and 220-kD isoforms.

Antibody 3, a rabbit polyclonal antibody, was prepared using a synthetic peptide (TSEKNPQDEQC) coupled to a carrier protein (inject maleimide activated bovine serum albumin; Pierce Chemical Co., Rockford, IL) through the cysteine residue at the carboxy terminus as antigen and affinity purified using a column of the recombinant protein corresponding to the region 2, which includes the ten amino acid sequence, coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ).

Cell Culture

Cerebellar cells were prepared from neonatal Wistar rats within 24 h after birth, plated onto poly-lysine–coated dishes or glass coverslips, and maintained in serum-free medium as described previously (Kunimoto et al., 1992).

Gel Electrophoresis and Immunoblot Analysis

Rat cerebella were dissected from Wistar rats of various ages and homogenized in 9 vol of 0.32 M sucrose containing 1 mM EGTA, pH 7.4, 1 mM NaN_3, 10 μg/ml pepstatin A, leupeptin, and 0.01% DFP. The homogenates were mixed with SDS-sample buffer and heated at 65°C for 20 min. Samples were electrophoresed on SDS-polyacrylamide gradient gels and were immunoblotted using ^{125}I-protein A to visualize antibodies as described by Davis and Bennett (1983) after electrophoretic transfer to Immobilon P membrane (Millipore Corp., Bedford, MA).

Cultured cells on plastic plates were washed once with Dulbecco's phosphate-buffered saline, mixed with SDS-sample buffer, scraped off from the plates, and heated at 65°C for 20 min. Samples were electrophoresed and immunoblotted in the same way.

Immunocytochemical Procedures

Cells grown on coverglasses coated with poly-L-lysine were fixed with 4% formaldehyde for 15 min at room temperature and washed three times with PBS. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with 10% normal goat serum and 1% bovine serum albumin in PBS for 30 min at room temperature. The coverglass was then incubated with 4 μg/ml rabbit polyclonal antibodies against brain ankyrin isoforms and mouse monoclonal antibodies against MAP2, GFAP, or tau in the presence of 0.05% Triton X-100 overnight at 4°C and washed five times with PBS containing 0.1% Tween 20. Ig molecules were visualized with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG.

Cryosections (10 μm in thickness) of cerebellum were prepared from adult rats as described before (Kunimoto et al., 1991) and immuno-stained in the same way.

Results

Preparation of an Antibody Specific to 220-kD Ankyrin$_B$

An antibody specific to 220-kD ankyrin$_B$ has not been successfully prepared by standard strategies using recombinant proteins as antigens, because this isoform is totally subsumed within the 440-kD isoform (Fig. 1). Therefore, a synthetic peptide corresponding to the splice site (10 amino acids, TSEKNPQDEQ, corresponding to the splice site plus an artificial C at the carboxy terminus for coupling to bovine serum albumin, Fig. 1, region 3) was designed to prepare polyclonal antibodies which can recognize the 220-kD isoform specifically. Such an antibody (antibody 3), raised against the synthetic peptide specifically recognized 220-kD ankyrin$_B$ by Western blot analysis of rat cerebellum, while an antibody against region 1 in Fig. 1 (antibody 1) recognized 440-kD ankyrin$_B$ specifically and an antibody against region 2 (antibody 2) recognized both 440- and 220-kD isoforms (Fig. 2).

Differential Expression of Two Brain Ankyrin Isoforms in Developing Cerebellar Cells

During the course of cerebellar development in rats, 440-kD ankyrin$_B$ appeared first and reached maximal expression 10–15 d after birth, while 220-kD ankyrin$_B$ appeared 10 d after birth and attained maximal expression only in adult rats (Fig. 3). In primary cerebellar cells, the expression of the two ankyrin$_B$ isoforms was regulated in a manner similar to that in vivo; the larger isoform appeared first and reached maximal expression after 9-d culture in vitro,

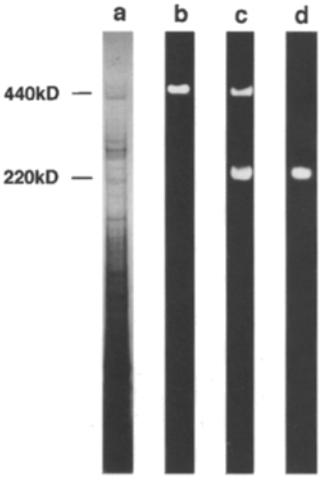


Figure 2. Specificity of antibodies. Western blot analyses of rat cerebellum (28-d old) were performed using antibodies 1 (*b*), 2 (*c*), or 3 (*d*). Coomassie blue–stained gel run in parallel with those used for immunoblotting was also shown (*a*).

1. *Abbreviations used in this paper*: DFP, diisopropyl fluorophosphate; DIV 9, 9 days in vitro; GAP, growth-associated protein; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2.

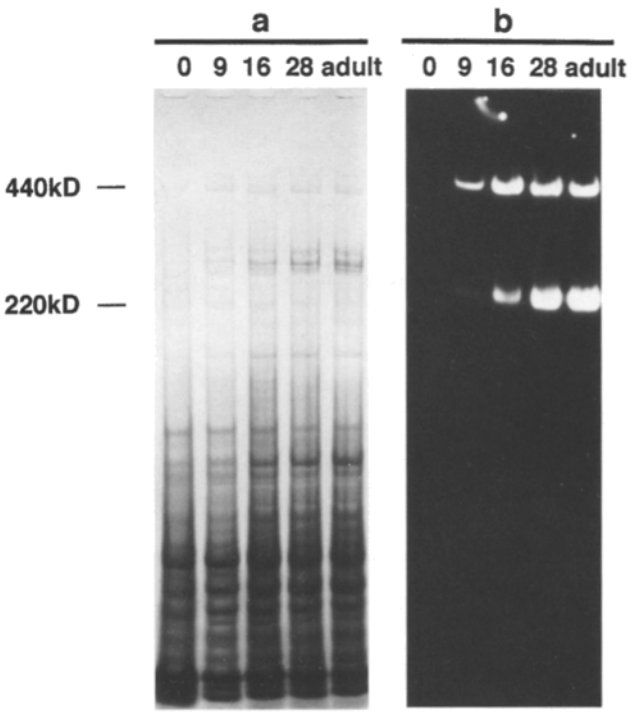


Figure 3. Expression of brain ankyrin isoforms in developing rat cerebellum. Western blot analyses of cerebellum from 1-, 9-, 16-, 28-d old and adult rats were performed using antibody 2 (*b*). Coomassie blue stained gel run in parallel with those used for immunoblotting was also shown (*a*).

while the smaller isoform appeared later and reached maximal expression only after 13-d culture in vitro or later (Fig. 4). At 18 d in vitro (DIV 18), 440-kD ankyrin$_B$ decreased significantly, but MAP2 also decreased in the same way, indicating that cerebellar neurons were dying out after prolonged culture period. The expression of 440-kD isoform is most active during the period of axogenesis both in vivo and in vitro, suggesting that this ankyrin isoform is closely related to the axogenesis.

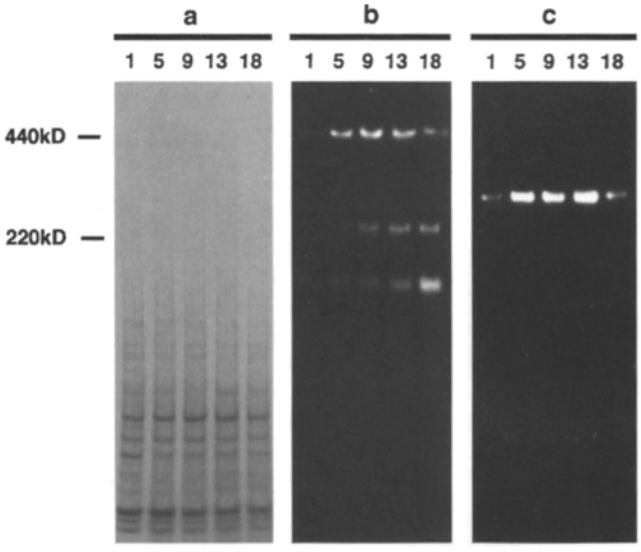


Figure 4. Expression of brain ankyrin isoforms in rat cerebellar cells in primary culture. Western blot analyses of cerebellar cells cultured for 1, 5, 9, 13, 18 d in vitro were performed using antibody 2 (*b*) or monoclonal antibody against MAP2 (*c*). Coomassie blue–stained gel run in parallel with those used for immunoblotting was also shown (*a*).

It is noteworthy that a 150-kD protein was also expressed after prolonged culture period, when neuronal cells were dying out (Fig. 4). Although this isoform of ankyrin has not yet been well characterized (Otto et al., 1991; Kunimoto et al., 1991) and was not expressed so much in normal cerebellum (Fig. 3), 150-kD ankyrin may be a nonneuronal isoform of brain ankyrin.

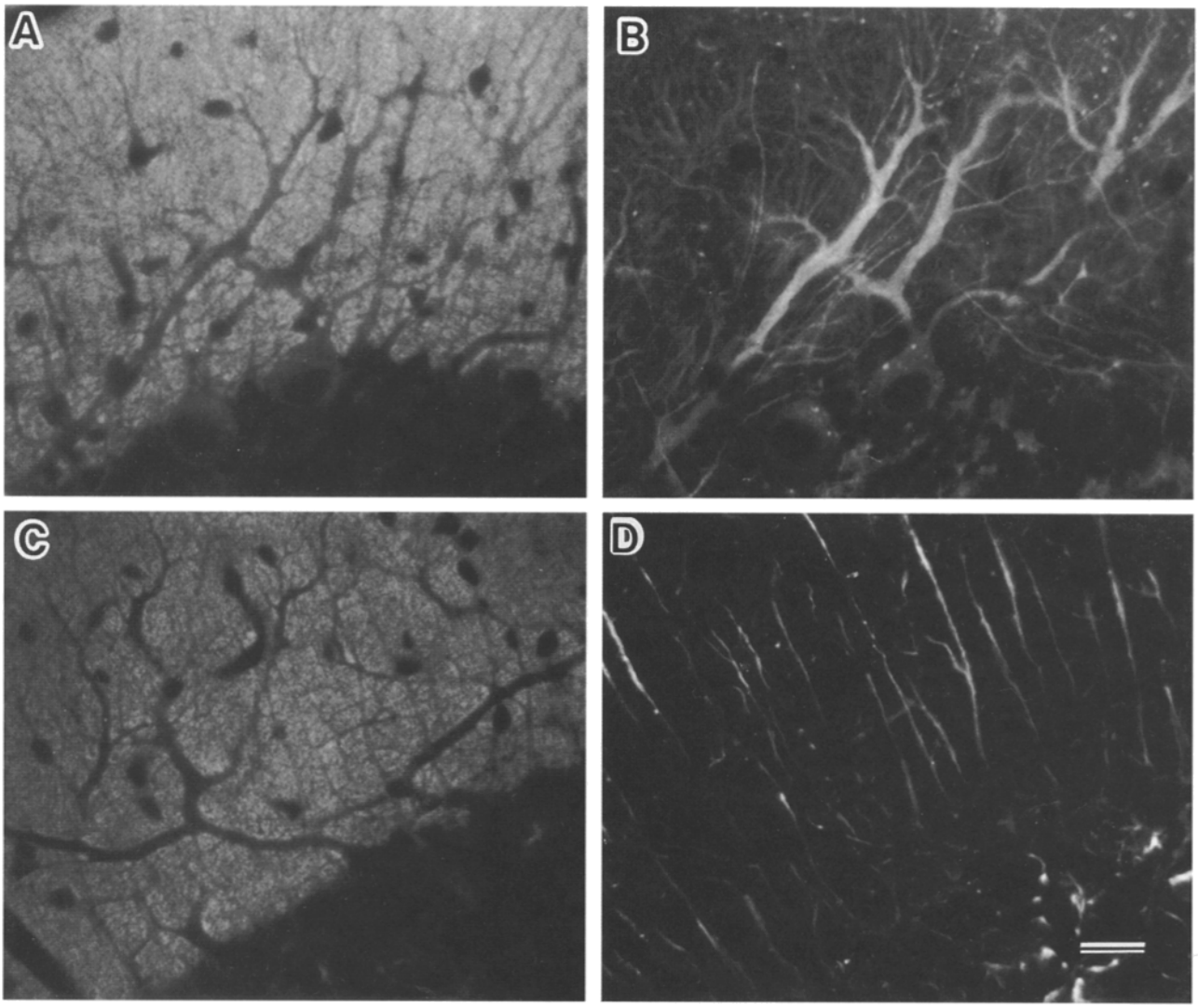
Immunocytochemical Localization of Brain Ankyrin Isoforms in Rat Cerebellum

In cryosections of adult rat cerebellum stained with double-label immunofluorescence, 440-kD ankyrin$_B$ was localized in the molecular layer which is comprised of mainly parallel fibers from granular neurons, dendrites from Purkinje cells, and Bergman glias (Paley and Chan-Paley, 1974) (Fig. 5, *A* and *C*). The staining of 440-kD ankyrin$_B$ did not overlap with that of MAP2 (the dendrites and cell bodies of Purkinje cells were stained, Fig. 5 *B*) (Bernhardt and Matus, 1984) or with that of GFAP (Bergman glias were stained, Fig. 5 *D*) (Schachner et al., 1977), showing that 440-kD ankyrin$_B$ is localized in the parallel fibers in the molecular layer. Furthermore, the staining of 440-kD ankyrin$_B$ overlapped quite well with that of tau in molecular layer and in white matter at unmyelinated stage of 16-d-old rats (Fig. 5, *E* and *F*). In contrast, stronger staining of 220-kD ankyrin$_B$ was observed in the dendrites and cell bodies of Purkinje cells (Fig. 5 *G*), which is overlapping with that of MAP2 (Fig. 5 *H*), in addition to general weak staining.

Immunocytochemical Localization of Brain Ankyrin Isoforms in Cerebellar Cells in Primary Culture

In primary cerebellar cells at 9 d in vitro (DIV 9), 440-kD ankyrin$_B$ was colocalized with tau in the axons (Fig. 6, *A* and *B*) stained by double-label immunofluorescence. Conversely, the staining of 440-kD ankyrin$_B$ was complementary to that of MAP2 (Fig. 6, *D* and *E*, typical site is indicated by *arrows*), which was localized to the dendrites and cell bodies of neurons (Fig. 6 *E*). Stronger staining of 440-kD ankyrin$_B$ was observed at the fasciculated axons (Fig. 6 *G*). It is noteworthy that 440-kD ankyrin$_B$ was concentrated at the tip of growing neurites in primary cerebellar neurons at DIV 1 (Fig. 7, *A*, *D*, and *G*, indicated by *arrows*). At this stage, the staining of 440-kD ankyrin$_B$ was also complementary to that of MAP2 (Fig. 7 *B*) and was overlapping with that of tau, except that tau was not necessarily concentrated at the tip of neurites (Fig. 7 *E*). However, GAP-43, a well-established marker for the axonal growth cone (Goslin et al., 1988; Liu et al., 1991), was not expressed very much and was not necessarily concentrated at the tip of growing neurites in the present culture system (Fig. 7 *H*).

In contrast to 440-kD ankyrin$_B$, the staining of the 220-kD isoform was quite similar to that of MAP2 (Fig. 8, *A* and *B*). In addition, 220-kD ankyrin$_B$ was localized in astroglial cells which were not stained with anti-MAP2 anti-



body (Fig. 8, *A* and *B*, indicated by *arrows*), but stained with anti-GFAP antibody (Fig. 8, *D* and *E*).

Discussion

This report describes the differential expression and localization of two alternatively spliced isoforms of brain ankyrin, 440- and 220-kD ankyrin$_B$, in rat neural cells. 440-kD ankyrin$_B$ has been suggested to localize in unmyelinated or premyelinated axons by using specific antibodies raised against its unique insert (Kunimoto et al., 1991; Chan et al., 1993). However, the localization of 220-kD ankyrin$_B$ has not been clearly demonstrated, because this isoform is totally subsumed within the 440-kD isoform (Fig. 1), which prevents successful production of specific antibodies by standard strategies using recombinant proteins as antigens. The only unique sequence for the 220-kD isoform is the stretch corresponding to the junction between the spectrin-binding domain and the COOH-terminal domain.

In this study, a peptide of ten amino acids including the junction (Fig. 1, region *3*) was synthesized and used as an antigen to produce polyclonal antibodies. Such an antibody, antibody 3, recognized the 220-kD isoform specifically on the immunoblot, while antibody 1 recognized the 440-kD isoform specifically and antibody 2 did both isoforms (Fig. 2). Although the peptide sequence of the antigen is based on human brain ankyrin cDNA sequence, antibody 3 can detect the 220-kD isoform of rat brain ankyrin, suggesting that rat brain ankyrin has the same amino acid sequence corresponding to the junction. These antibodies revealed the differential expression and localization of the two brain ankyrin isoforms in rat cerebellar cells.

Both Isoforms were expressed in primary cerebellar cells in a manner similar to that in vivo; the larger isoform expressed maximally when axogenesis is actively conducted, while the smaller isoform came up later (Figs. 3 and 4). It is noteworthy that the 220-kD isoform did not decrease in parallel with MAP2 (Fig. 4) at DIV 18 and was also expressed in astroglial cells (Fig. 8), suggesting that the increase in the amount of this isoform is contributed not only by neurons but also by glial cells.

440-kD ankyrin$_B$ was localized in the axons of cerebellar neurons both in vivo (Fig. 5) and in vitro (Fig. 6) using antibody 1, while the 220-kD isoform was rather localized in the cell bodies and dendrites of neurons using antibody 3 (Figs. 5 and 8).

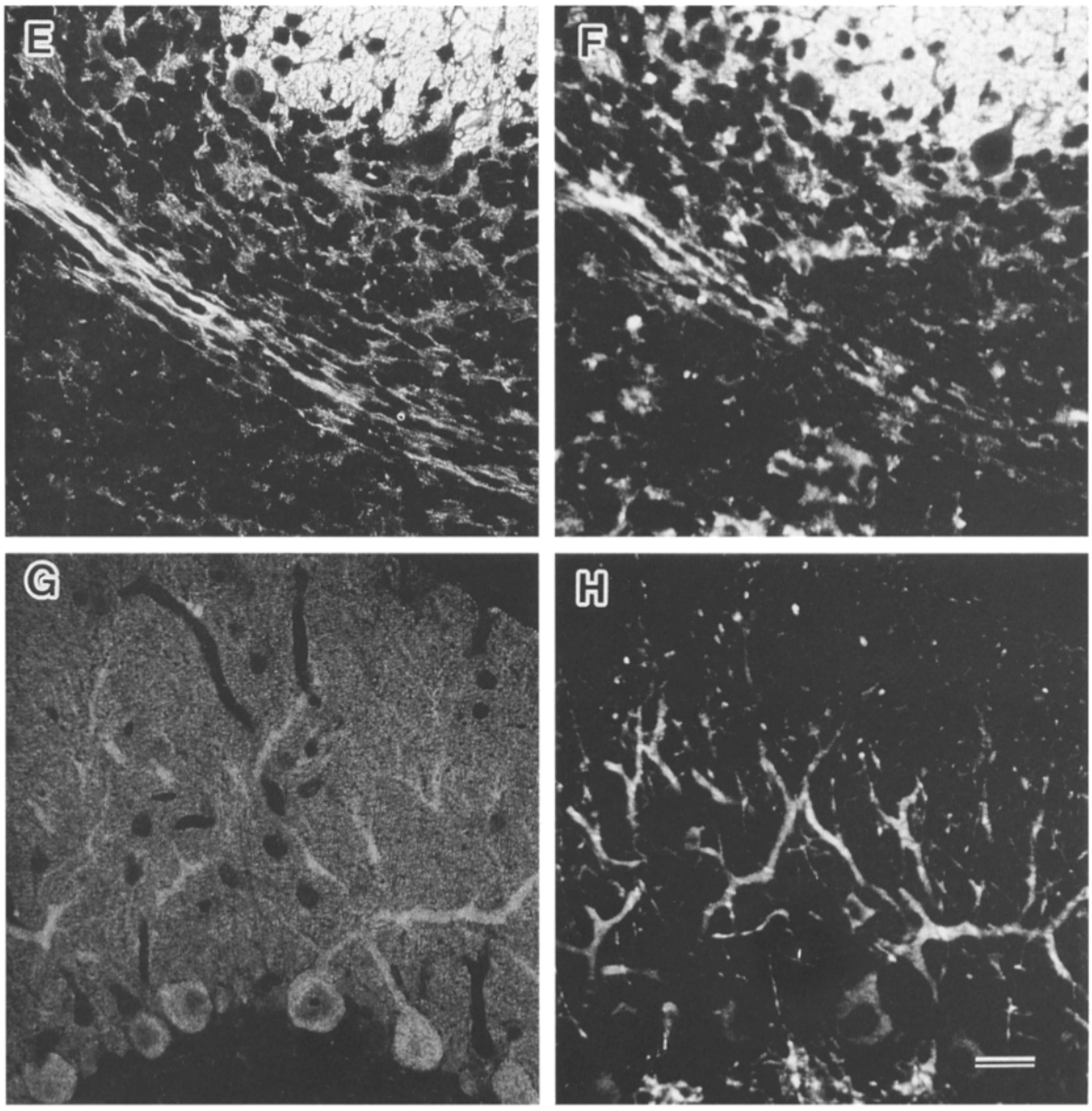


Figure 5. Immunocytochemical localization of 440- and 220-kD ankyrin$_B$ in cerebellum from adult rats. Cryosections of cerebellum from adult rat (*A–D*, *G*, and *H*) or 16-d-old rat (*E* and *F*) were stained with double-label immunofluorescence using antibody 1 (*A*, *C*, and *E*) or antibody 3 (*G*), and monoclonal antibodies against MAP2 (microtubule-associated protein 2) (*B* and *H*), GFAP (glial fibrillary acidic protein) (*D*) or tau (*F*). Micrographs in *E–H* were taken under a confocal microscope (MRC-1024; BioRad). Bar, 20 μm.

Based on these results, it is concluded that 440-kD ankyrin$_B$ is a neuron-specific isoform, while 220-kD ankyrin$_B$ is rather general in neural tissue and is expressed both in neurons and glial cells. In addition, in neuronal cells 440-kD ankyrin$_B$ is sorted to the axons, while the 220-kD isoform is abundant in the dendrites and cell bodies.

To date, only a few proteins, such as GAP-43 (Goslin et al., 1988; Liu et al., 1991) and certain isoforms of tau (Binder et al., 1986), have been shown to be targeted to axons, but the sorting mechanism has not been elucidated. Although tau is abundant in the axons and MAP2 is localized in the dendrites/cell bodies (Bernhardt and Matus, 1984), they share a homologous microtubule binding domain in their COOH-terminal halves (Aizawa et al., 1988; Lee et al., 1988; Lewis et al., 1988). So far, several possible mechanisms for the selective localization of tau in the axon and MAP2 in the dendrites have been suggested; first is the locally differing microtubule-binding affinities of the two proteins (Kanai and Hirokawa, 1995), second is the decreased transit of MAP2 in the axons (Kanai and Hirokawa, 1995), third is the selective stabilization of MAP2 in the dendrites and tau in the axons (Okabe and Hirokawa, 1989), and fourth is the extended localization of mRNA in the dendrites (Garner et al., 1988).

Furthermore, MAP2C, embryonic isoform of MAP2 produced by alternative splicing, has been shown to lack the cross-linking sidearm sequences and dendritic targeting signal of MAP2, resulting in uniform localization in the axon, cell body and dendrite (Papandrikopoulou et al., 1989).

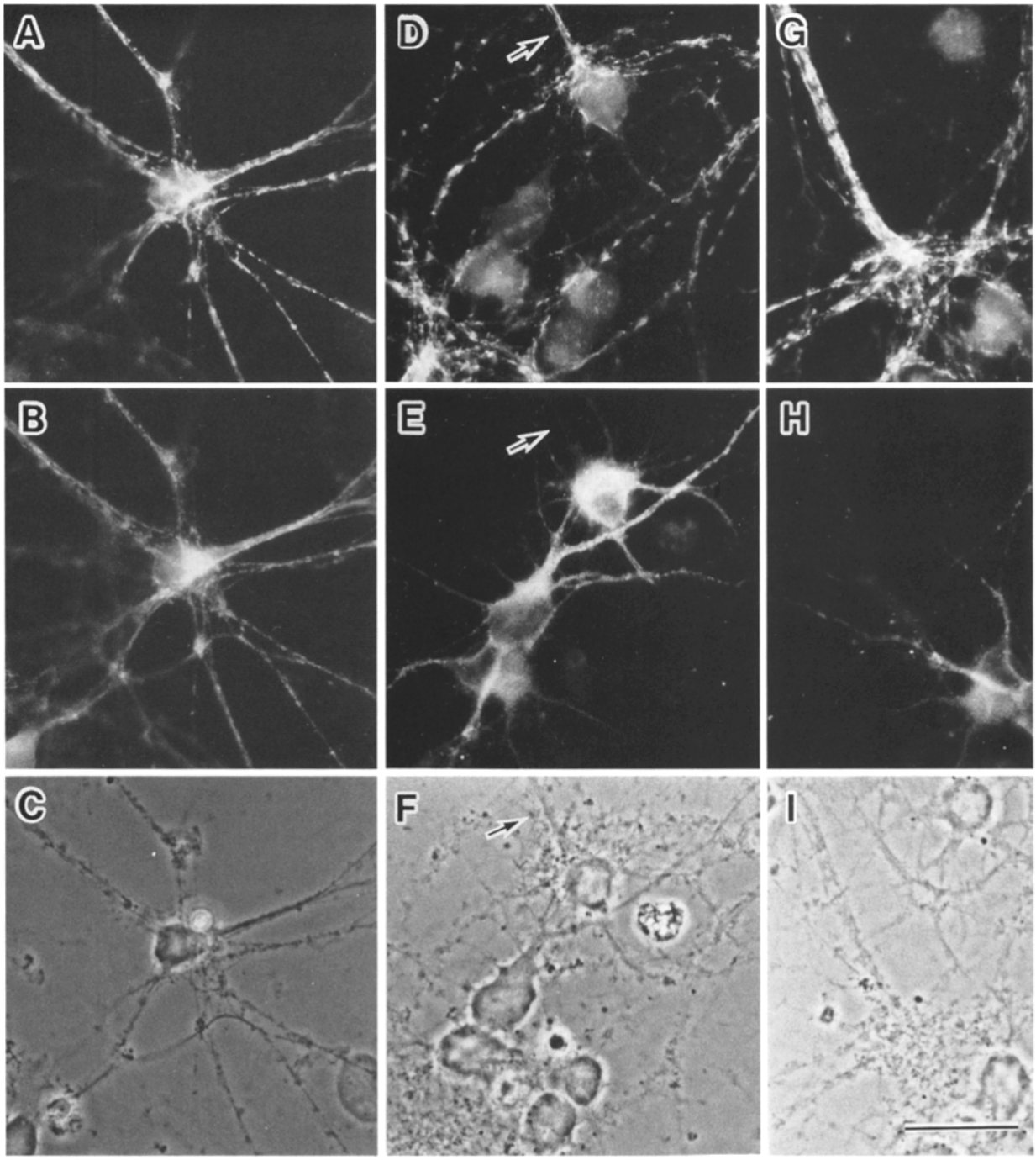


Figure 6. Immunocytochemical localization of 440-kD ankyrin$_B$ in primary cerebellar cells of rats. Cerebellar cells cultured for 9 d were stained with double-label immunofluorescence using antibody 1 (*A*, *D*, and *G*) and monoclonal antibodies against tau (*B*) or MAP2 (*E* and *H*), simultaneously. Corresponding phase contrast micrographs (*C*, *F*, and *I*) are also shown. Bar, 20 μm.

In case of the two brain ankyrin isoforms, the 220-kD isoform is abundant in the dendrites/cell bodies and the 440-kD isoform is localized in the axons, although they share the same membrane binding and spectrin binding domains (Fig. 1). 220-kD ankyrin$_B$ can be regarded as a naturally occurring deletion mutant of 440-kD ankyrin$_B$ lacking the 220-kD inserted domain and is not sorted to the axons. Furthermore, the 220-kD inserted domain contains several short stretches of sequence similarity with GAP-43, suggesting the presence of a common mechanism for the targeting of these axonal proteins (Chan et al., 1993). Taken together, it is strongly suggested that the 220-kD inserted domain plays an essential role for the sorting of 440-kD ankyrin$_B$ to the axons.

The membrane binding domain of brain ankyrin can bind to the cytoplasmic domains of neurofascin/L1/Nr-CAM family of nervous system cell adhesion molecules (Davis and Bennett, 1994). Among them L1 has been shown to be localized in the molecular layer of the cerebellum (Linder et al., 1983), which is quite similar to the

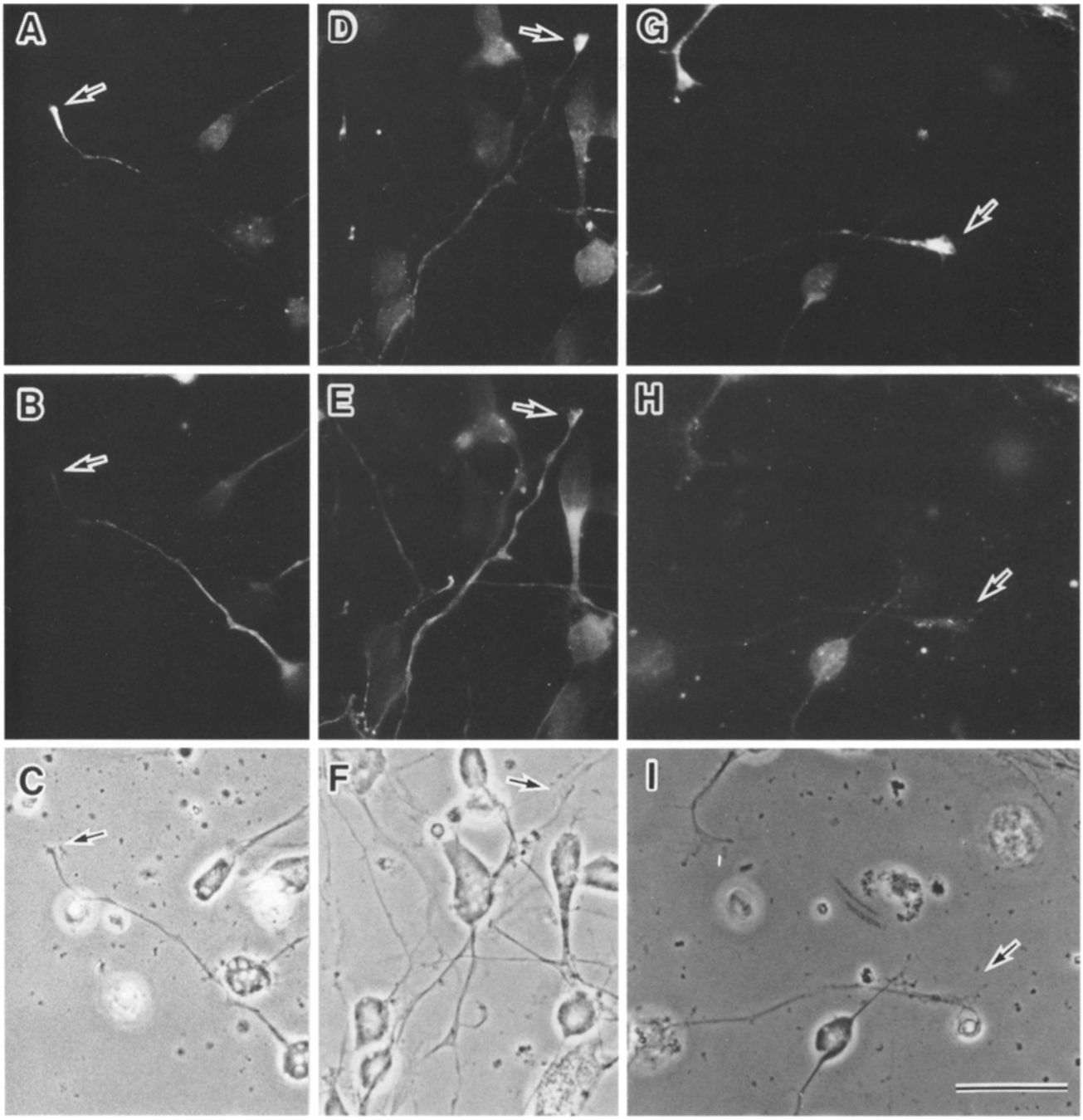


Figure 7. Immunocytochemical localization of 440-kD ankyrin$_B$ in primary cerebellar cells of rats. Cerebellar cells cultured for 1 d were stained with double-label immunofluorescence using antibody 1 (*A*, *D*, and *G*) and monoclonal antibodies against MAP2 (*B*), tau (*E*), or GAP-43 (*H*), simultaneously. Corresponding phase contrast micrographs (*C*, *F*, and *I*) are also shown. Bar, 20 μm.

localization of 440-kD ankyrin$_B$ (Fig. 5). It is, therefore, possible that the cytoplasmic domains of integral membrane proteins like L1 are involved in the differential localization of the two brain ankyrin isoforms. In that case, however, modifying role of the inserted domain must be essential for the differential sorting, because the two brain ankyrin isoforms share the same membrane binding domain.

At the earlier stage of neurite extension from cerebellar neurons in primary culture like DIV 1, only a little amount of 440-kD ankyrin$_B$ was expressed but was highly concentrated at the growing tip of the neurites (Fig. 7), which is consistent with a previous observation that the expression of 440-kD ankyrin$_B$ is upregulated by the induction of neurite extension and this isoform is highly concentrated at the tip of growing neurites together with GAP-43 in human neuroblastoma NB-1 cells (Kunimoto, 1995). Although 440-kD ankyrin$_B$ was not well colocalized with GAP-43 in the present culture system, these facts may indicate that 440-kD ankyrin$_B$ plays an important role at the growth cone.

Thus ankyrin$_B$ provides an interesting example of alternative splicing in neurons which produces protein isoforms

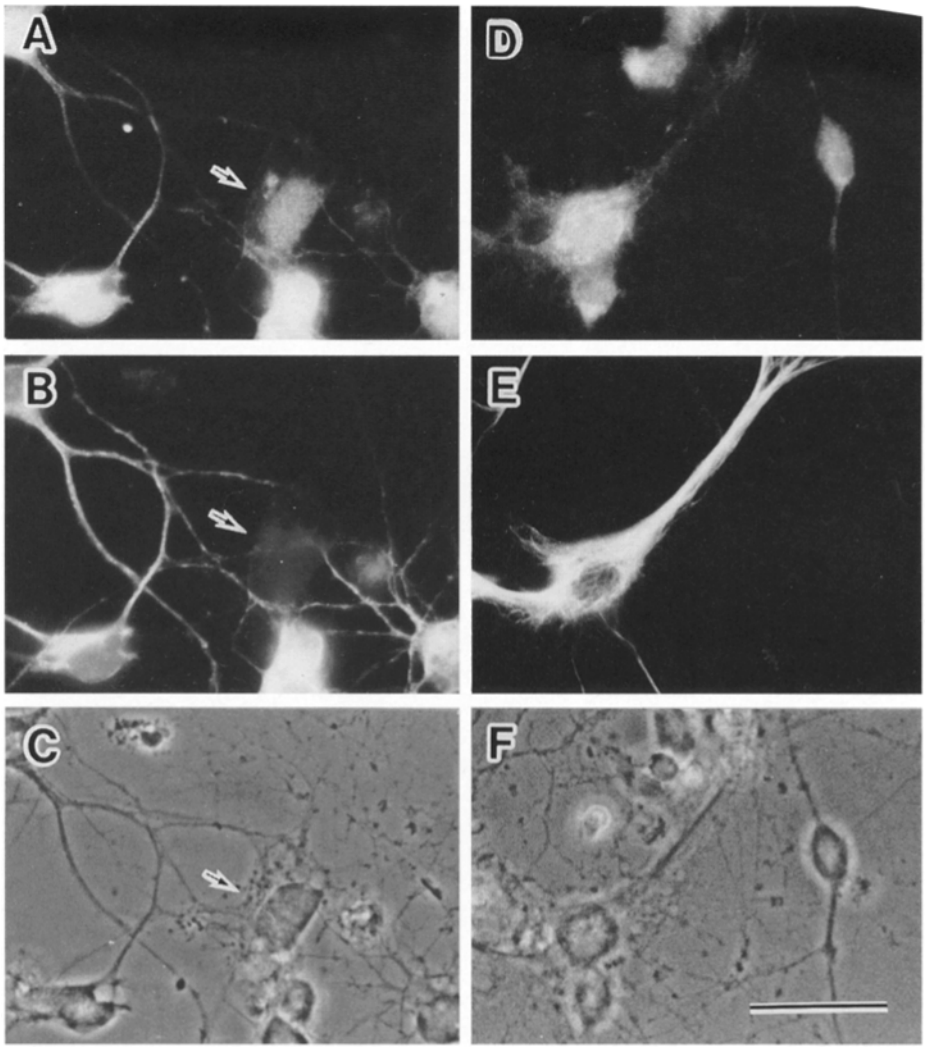


Figure 8. Immunocytochemical localization of 220-kD ankyrin$_B$ in primary cerebellar cells of rats. Cerebellar cells cultured for 9 d were stained with double-label immunofluorescence using antibody 3 (*A* and *D*), and monoclonal antibodies against MAP2 (*B*) or GFAP (*E*), simultaneously. Corresponding phase contrast micrographs (*C* and *F*) are also shown. Bar, 20 μm.

differentially targeted to axons and dendrites/cell bodies. In addition, ankyrin$_G$, another member of ankyrin family expressed in neuronal cells, is targeted to nodes of Ranvier and the initial segments of axons (Kordeli et al., 1991, 1995) and also have a unique insert similar to that of 440-kD ankyrin$_B$, which may be responsible for the targeting. Ankyrins will perhaps be a useful model for investigating protein sorting in neurons.

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