J. Biochem. 122, 1146-1152 (1997)

Isolation and Characterization of a 230 kDa Protein (p230) Specifically Expressed in Fetal Brains: Its Involvement in Neurite Outgrowth from Rat Cerebral Cortex Neurons Grown on Monolayer of Astrocytes[1]

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Received for publication, July 7, 1997

By screening with monoclonal antibodies (mAbs) raised against growth cone membrane fraction from fetal porcine brains, we have identified a 230 kDa antigen, termed p230. Western blot analysis of extracts from various tissues demonstrated that p230 is specifically expressed in brains, in which its expression is temporally restricted; it was especially prominent in the embryonic and the early postnatal stage, and decreased to subdetectable levels in the adult brain. Further characterization of p230 revealed that it is a peripherally-membrane associated, cell surface protein produced by astrocytes. Neurite outgrowth of E18 rat cerebral cortex neurons cultured on a monolayer of astrocytes was significantly reduced in the presence of anti-p230 polyclonal antibody. Partial amino acid sequences of p230 purified from fetal porcine brains were highly homologous to an extracellular matrix protein, tenascin-C. These lines of evidence suggest that p230, a tenascin-C-like molecule present in fetal porcine brains, plays important roles during early brain development, particularly in growth cone guidance.

Key words: astrocyte, cerebral cortex, extracellular matrix, neurite outgrowth, tenascin-C.

For the nervous system to function properly, neurons must accurately find their targets and make the right connections. During the early stage of brain development, the growth cones appear at the tip of axons and navigate them correctly using guidance cues in their outside environment (*1*). It is clear that both positive and negative signals control this process, such that the growth cones recognize cells and axon bundles and then choose to join or avoid them (*2-5*). These processes are mediated by cell surface molecules that exist on the growth cone membrane. For example, cell adhesion molecules of the immunoglobulin and cadherin superfamilies constitute important regulatory components (*6*). On the other hand, inhibitory and anti-adhesive molecules have also been implicated in growth cone guidance (*4, 7*). In addition, glycoproteins of the extracellular matrix (ECM) and their receptors are involved in the control of axon elongation (*8*).

Among ECM proteins, tenascin glycoproteins have attracted particular attention because they are synthesized at specific locations and at crucial times during development. In the central nervous system (CNS), tenascin-C is transiently expressed by immature astrocytes and down-regulated in most regions of the CNS after neuritogenesis has proceeded (*9, 10*). Thus, tenascin-C has been implicated in several key events of neuritogenesis such as neuronal migration and neurite outgrowth, and anti-repulsive properties for neuronal cell bodies and growth cones have also been attributed to the glycoprotein.

In this study, we aimed initially to isolate the molecules on the growth cone membrane that are involved in neurite outgrowth and/or target recognition. For this purpose, a panel of hybridoma lines were obtained from mice immunized with a fraction that was enriched with growth cone particles (GCPs) prepared from fetal porcine brains. Subsequently, hybridomas which produced antibodies against antigens present only in the fetal brains were selected. Further investigations with one clone which recognized a 230 kDa protein, named p230, revealed that this protein is expressed only in the fetal brains and is involved in neurite outgrowth from cortical neurons grown on the monolayer of astrocytes.

MATERIALS AND METHODS

Animals—Fetal and adult pigs were obtained from the Shibaura Zoki, Tokyo. BALB/c mice and Wistar rats were purchased from Sankyo Labo Service, Tokyo.

Cell Culture—Cerebral cortexes from 18-day gestation fetal rats were removed and placed into a Petri dish containing Dulbecco's modified Eagle's medium (DMEM). The meningeal tissue and as much white matter as possible

[1] This work was supported by a Grant-in-Aid for Scientific Research (No. 08760069) from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GCP, growth cone particle; HRP, horseradish peroxidase; mAb, monoclonal antibody; PEI, polyethyleneimine; SDS, sodium dodecyl sulfate; SS, synaptosome; TBS, Tris-buffered saline.

were removed. The remaining tissue was finely minced with dissecting knives, then treated with papain for 10 min at 37°C. The dissociated cells were pelleted and resuspended in DMEM supplemented with 10% fetal calf serum (FCS). DNase I was added and incubated for 10 min at 37°C, and the clusters of cells were triturated several times. The cell suspension was passed through a fine mesh. For neuronal cell culture, the cells suspended in DMEM containing 5% each of FCS and horse serum (HS) supplemented with insulin and transferrin (5 μg/ml each) were plated into polyethyleneimine-coated 96-well plastic plates and incubated at 37°C in 10% CO_2 in humidified air. Cultures were maintained for 3 to 7 days. For the cell culture of astrocytes, cell suspension prepared as above was placed into 10-cm non-coated plastic dishes. Once the astrocytes reached confluence (after approximately 10 days to 2 weeks), the plates were washed to remove loosely adherent, contaminating top cells. The cells were grown in DMEM supplemented with 10% FCS at 37°C in 10% CO_2 in humidified air.

Preparation of Growth Cone Particles (GCPs)—GCPs were isolated from fetal pig brain as described by Pfenninger *et al.* (*11*) with modifications. Briefly, fetal pig brains were homogenized in 8 volumes of 0.32 M sucrose with 5 mM HEPES buffer at pH 7.4 containing protease inhibitors (0.5 mM PMSF, 1 μg/ml each of antipain and leupeptin and 0.5 mM EDTA). After centrifugation at 1,660 × *g* for 10 min, the low speed supernatant was layered onto a discontinuous sucrose gradient and centrifuged at 100,000 × *g* for 60 min at 4°C. The 0.32 M sucrose/0.75 M sucrose interface was recovered and pelleted at 39,800 × *g* for 30 min at 4°C. This GCP pellet was resuspended in PBS.

Preparation of Synaptosomes (SSs)—SSs were prepared from adult pig brain essentially according to the procedure of Cohen *et al.* (*12*) with modification. Adult pig brains were homogenized in 8 volumes of 0.32 M sucrose with 5 mM HEPES buffer at pH 7.4 containing protease inhibitors. After centrifugation at 750 × *g* for 10 min, the supernatant was centrifuged at 17,300 × *g* for 10 min. The pellet was homogenized in 0.32 M sucrose plus 1 mM $NaHCO_3$ and layered onto a three-step sucrose density gradient (1.2 M; 1.0 M; 0.85 M). This gradient was spun at 100,000 × *g* for 60 min. The band formed at the 1.0/1.2 M sucrose interface was collected and pelleted for 30 min at 48,200 × *g*. The pelleted SSs fraction was resuspended in PBS.

Differential Screening of Hybridoma—Selected wells were screened for the presence of antibodies that would differentially recognize GCPs but not SSs in an enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated overnight at 4°C with 0.4 mg of GCPs or SSs in PBS. They were blocked with 5% skim milk in Tris-buffered saline (TBS; 15 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated with supernatants from selected hybridoma wells. After washing with saline, the plates were incubated with an anti-mouse IgG conjugated with horseradish peroxidase. The plates were again washed with saline, then reacted with *o*-phenylenediamine (Sigma). The reaction was terminated by adding 3% H_2SO_4. Hybridomas of interest were cloned twice by the method of limiting dilutions. Ascite fluids were produced by injecting 10^7 cells into pristane-primed BALB/c mice and collected 1 week later.

Treatment of Membrane Fractions with High Salt and Alkaline Buffer—A homogenate was prepared from fetal porcine brains in HEPES buffer at pH 7.4 containing 0.32 M sucrose and protease inhibitors. After centrifugation at 1,500 × *g* for 15 min, the pellet was discarded and the supernatant was separated into a soluble cytosol fraction and a membrane pellet by ultracentrifugation at 100,000 × *g* for 60 min. These membrane pellets were resuspended and incubated in 1 M NaCl, 0.1 M Na_2CO_3 (pH 11.0) or 1% Triton X-100 for 40 min at 4°C, followed by ultracentrifugation at 100,000 × *g* for 60 min. The resulting pellet was resuspended in Tris-buffer in the same volume as the supernatant. These pellets and supernatants were analyzed by Western blotting using anti-p230 mAb as a probe.

Preparation of Tissue Lysates—Crude cell extracts used for immunoblot experiments were prepared as follows: Tissues were removed and homogenized in HEPES buffer at pH 7.4 containing 0.32 M sucrose and protease inhibitors. The homogenates were centrifuged for 15 min at 1,500 × *g* at 4°C, the pellet was discarded and the supernatant was then analyzed by SDS-PAGE and immunoblotting. Protein concentration was measured by the method of Lowry (*13*), using bovine serum albumin as standard.

Gel Electrophoresis and Immunoblotting—SDS-PAGE was performed on 7.5% acrylamide gels under reducing conditions. Gels were stained with Coomassie brilliant blue. For Western blotting, the separated proteins were transferred to a nitrocellulose membrane using a semi-dry blotter. The blotted membrane was blocked with 5% skim milk in TBS for 30 min, then incubated with the primary antibodies for 1 h. The membrane was reacted with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:250 dilution; Vector), then developed in 4-chloro-1-naphthol or 3,3′-diaminobenzidine tetrahydrochloride (DAB) with nickel enhancement.

Affinity Purification of p230—Immunoaffinity purification of p230 was performed as described by Schneider *et al.* (*14*). The anti-p230 mAb, purified from ascite fluids using MAb Trap G II (Pharmacia), was coupled to protein G-Sepharose CL-4FF (15 mg/3 ml packed beads; Pharmacia). Antibody was then cross-linked to the beads using dimethypimelimidate (Sigma). A homogenate, prepared from fetal porcine brain in HEPES buffer at pH 7.4 containing 0.32 M sucrose and protease inhibitors, was centrifuged for 15 min at 1,500 × *g* at 4°C. The pellet was discarded and the supernatant was centrifuged at 100,000 × *g* for 60 min. The pellet was resuspended for 30 min in TBS containing 1% NP-40 and then centrifuged at 100,000 × *g* for 60 min. The supernatant, a crude membrane fraction, was applied to the antibody-coupled column. Unbound proteins were washed off the column with (1) 0.5 M NaCl, 0.05 M Tris-HCl (pH 8.2), 1 mM EDTA, 0.5% NP-40; (2) 0.15 M NaCl, 0.05 M Tris-HCl (pH 8.2), 0.5% NP-40, 0.1% SDS; (3) 0.15 M NaCl, 0.1% SDS. p230 was eluted with 0.1 M diethylamine (pH 11.0), 0.1% SDS, 0.5% NP-40. The eluted sample was neutralized immediately with 1/10 volume of 0.2 M NaH_2PO_4.

Immunofluorescence Analysis—Astrocytes cultured on glass coverslips were fixed in 4% paraformaldehyde in PBS and washed in TBS, then treated with 0.3% Triton X-100 for 15 min. Non-specific binding sites were blocked with 5% skim milk in TBS for 30 min. The coverslips were incubated with anti-p230 polyclonal antibody diluted 1:100 in TBS

for 1 h at room temperature. After washing in TBS, the coverslips were incubated with rhodamine-conjugated goat anti-rabbit secondary antibody (Cappel) diluted 1:100 in TBS for 30 min at room temperature. After washing in TBS, the incubation and wash steps were repeated using anti-GFAP mAb (1:50 dilution; Boehringer Mannheim) and fluorescein-conjugated sheep anti-mouse secondary antibody. After the final incubation, coverslips were washed in TBS and mounted on microscope slides.

Morphological Evaluation of Neurite Outgrowth—E18 cerebral cortex neurons plated onto an astrocyte monolayer in a 24-well plate were cultured in the presence of anti-p230 polyclonal antibody (20 μg/ml) or non-immunized IgGs (20 μg/ml). After 48 h, the cells were fixed in paraformaldehyde in PBS and immunostained with anti-MAP-2 antibody (1:300 dilution; Sigma M-4403). Cells with neurites longer than twice the diameter of the cell body were counted in five randomly chosen fields and the percentages of such cells in the total numbers of cells were calculated.

Labeling of Cell Surface Proteins and Immunoprecipitation—Surface labeling of cerebral cortex neurons from fetal porcine brains with biotin was performed as described by Tsukita *et al.* (*15*). Cells isolated from fetal porcine brains were grown on 10-cm plastic dishes for 2 days. At ~70% confluence, the cells were washed three times with 0.1 M HEPES buffer at pH 8.0 containing 50 mM NaCl, then incubated for 15 min at room temperature with 2 ml of 0.1 M HEPES buffer at pH 8.0 containing 1 mg/ml sulfo-succinimidobiotin (sulfo-NHS-biotin) (Pierce Chemical), 50 mM NaCl and protease inhibitors. Cells were washed with DMEM followed by PBS three times, then processed for immunoprecipitation. In a control experiment, cells were lysed with RIPA buffer before biotinylation.

The labeled cells on one dish were lysed and incubated in 0.8 ml of RIPA buffer (0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and protease inhibitors) for 5 min. The RIPA lysate was removed from the dish after fully dislodging any remaining cellular debris from the plate surface with a rubber policeman. The lysate from one dish was incubated in a 1.5-ml microtube on ice for an additional 10 min, then clarified by centrifugation at 12,000$\times g$ for 20 min. The RIPA-soluble supernatant was immunoprecipitated with 20 μl of protein G-Sepharose 4B (Pharmacia) conjugated with either anti-p230 mAb or anti-tubulin mAb (Amersham N356). Sepharose 4B-bound immune complexes were washed six times with RIPA buffer. They were then eluted by boiling in sample buffer, resolved by SDS-PAGE and blotted onto the nitrocellulose membranes. To detect biotinylated proteins, the nitrocellulose membranes were soaked for 1 h with TBS containing 5% skim milk, followed by a 30-min incubation with avidin peroxidase. After washing in TBS, biotinylated proteins were visualized by the method described above.

Amino Acid Sequence Analysis of p230—The partially purified protein was separated by SDS-PAGE, and the protein band of p230 visualized by Coomassie staining was cut out. The protein was digested with lysylendopeptidase and the resulting peptide fragments were separated by reverse-phase high performance liquid chromatography using a gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid. The isolated peptides were subjected to automated Edman degradation using an ABI model 477A protein sequencer connected on-line to an ABI model 120A phenylthiohydantoin analyzer (Perkin-Elmer).

RESULTS

Identification of p230 as a Fetal Brain-Specific Protein—We obtained a panel of hybridoma lines from mice immunized with a fraction that was enriched with growth cone particles (GCPs) prepared from fetal porcine brains. The fusion of spleen and myeloma cells produced ~400 hybridomas. Antibodies secreted from these hybridomas were assessed by ELISA using GCP *versus* synaptosome (SS) proteins. Eighty hybridomas which exhibited higher reactivity against GCPs than SSs were selected and further analyzed. One clone which recognized a 230 kDa protein in Western blots of fetal porcine brain extracts was selected in the following studies, and this antigen was named p230. Brain extracts from adult and fetal pigs, fetal rat, and chick embryo were analyzed by immunoblotting using anti-p230 mAb. p230 was detected only in fetal porcine brain fraction, but not in adult brains or brain extracts from other animal species, namely, rat and chicken (data not shown). The distribution of p230 in various fetal porcine tissues other than brain, including heart, lung, liver, stomach, and kidney, was examined by immunoblot analysis (Fig. 1). Anti-p230 mAb did not recognize any immunoreactive bands in these tissues, indicating that p230 was present only in fetal porcine brain.

Biochemical Characterization of p230—Association of p230 with the membrane fraction was tested by a parallel immunoblot analysis of brain homogenate and the 100,000$\times g$ pellet and supernatant fractions. As shown in Fig. 2A, p230 was quantitatively recovered in the membrane pellet (lane 2). Solubilization of the membrane fraction with 100 mM Na_2CO_3 (pH 11.5) revealed that p230 was peripherally associated with the membrane or was resident in the lumen of the membrane vesicle (*16*). To further investigate the nature of association of p230 with the membrane, biotinylation of intact cells isolated from cerebral cortex of fetal porcine brains with sulfo-NHS-biotin was performed as described in "MATERIALS AND METHODS" (Fig. 2B). Since sulfo-NHS-biotin is membrane-impermeable, only proteins present on the cell

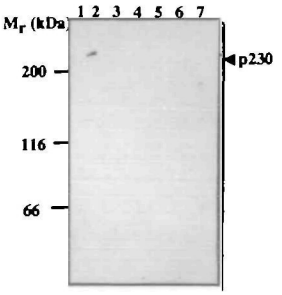

Fig. 1. **Fetal brain specific expression of p230.** Equal amounts of proteins extracted from adult pig brain or fetal pig tissues were separated by SDS-PAGE and subjected to Western blot analysis using anti-p230 mAb. The blotted membrane was developed with HRP conjugated with anti-mouse IgG(H+L). Lanes: 1, adult brain; 2, fetal brain; 3, heart; 4, lung; 5, liver; 6, stomach; 7, kidney. Anti-p230 mAb did not react with any tissues other than fetal brain.

surface should be labeled. After biotinylation, total cell lysates were prepared, immunoprecipitated with anti-p230 mAb, and then biotinylated proteins were detected by immunoblotting with avidin conjugated to horseradish peroxidase. In the control experiment, cells were lysed before labeling with sulfo-NHS-biotin, in which the total cellular proteins were biotinylated. A 230 kDa protein was detected in immunoprecipitates followed by immunoblotting before and after lysing the cells, indicating that p230 was present on the cell surface. In contrast, the intracellular protein tubulin was detected only after lysis, indicating that the labeling was indeed specific for proteins expressed on the surface of the plasma membrane. These sets of results demonstrated that p230 is a cell surface antigen and is peripherally associated with the plasma membrane.

Since the anti-p230 mAb did not recognize rat and chick proteins, we attempted to prepare a polyclonal antibody. Affinity-purification of p230 was performed from fetal porcine brain extracts as described in "MATERIALS AND METHODS," and the antiserum against purified p230 was raised in rabbits. The anti-p230 polyclonal antibody thus prepared recognized an approximately 230 kDa protein in fetal brain extracts from pigs, rats, and chicks, indicating that proteins homologous to p230 are also expressed in the animals other than pigs. The IgG fraction purified from this antiserum was used for further studies.

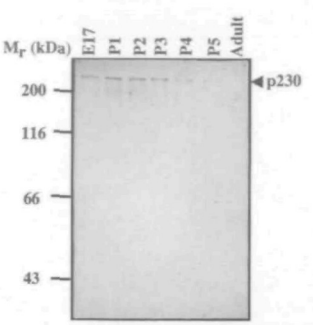

Fig. 3. **Expression of p230 during rat brain development.** p230 immunoreactivity was examined by Western blot analysis of rat brain lysates in various stages of development after SDS-PAGE separation. The intensity of the 230 kDa band was especially prominent in the embryonic and early postnatal stage, thereafter decreased, and was hardly detectable in the adult, which is consistent with the result obtained from the porcine brains. E17, embryonic day 17; P1, postnatal day 1; P2, postnatal day 2; P3, postnatal day 3; P4, postnatal day 4; P5, postnatal day 5.

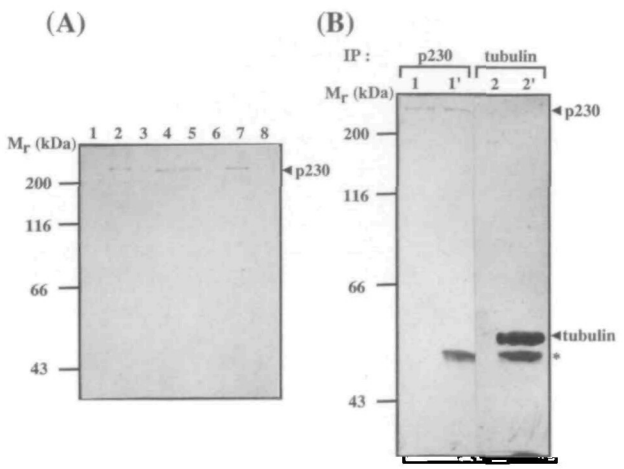

Fig. 2. **Biochemical properties and characterization of p230.** (A) After treatment with the various buffers, extracts of fetal pig brain were centrifuged at 100,000 × *g* to separate soluble and insoluble fractions. Equal amounts of proteins of each sample were separated by SDS-PAGE and analyzed by Western blot with anti-p230 mAb. The blotted membrane was developed with HRP conjugated anti-mouse IgG(H+L). p230 was solubilized with high pH (lane 5), suggesting that p230 was a protein associated peripherally with the membrane. Lanes: 1, TBS supernatant; 2, TBS pellet; 3, NaCl supernatant; 4, NaCl pellet; 5, Na_2CO_3 supernatant; 6, Na_2CO_3 pellet; 7, Triton X-100 supernatant; 8, Triton X-100 pellet. (B) Cells isolated from porcine brains were cultured for 2 days, and the cell surface proteins were labeled with sulfo-NHS-biotin as described in the "MATERIALS AND METHODS." In the control experiment, cells were lysed with RIPA buffer before biotinylation (lanes 1′ and 2′). These samples were immunoprecipitated with anti-p230 mAb (lanes 1 and 1′) or with anti-tubulin mAb (lanes 2 and 2′). Samples were separated by SDS-PAGE and transferred to nitrocelluose membranes, then the biotinylated proteins were detected by avidin conjugated HRP. A 230 kDa protein was detected before (lane 1) and after lysing the cells (lane 1′), whereas the intracellular protein tubulin was detected only after lysis (lane 2′). The asterisk marks biotinylated IgGs, which might be biotinylated because of inadequate quenching procedure.

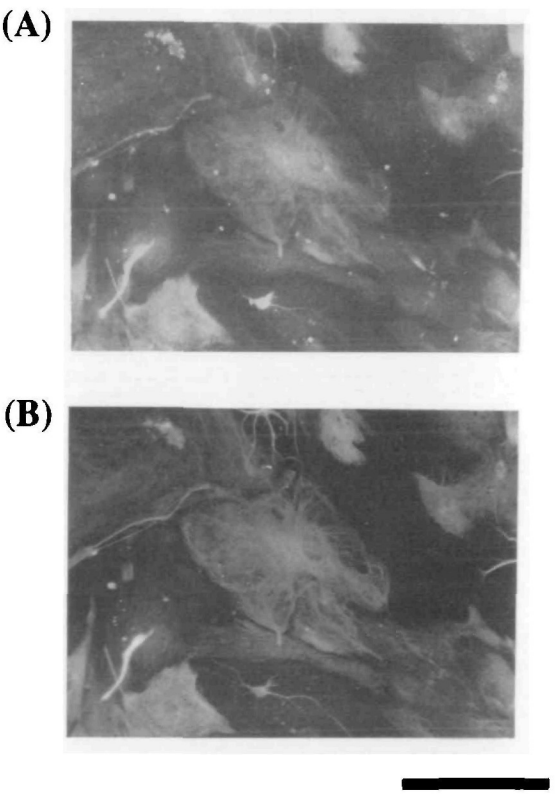

Fig. 4. **Immunocytological localization of p230 on astrocytes.** To determine the localization of p230, cerebral astrocytes were double labeled with anti-p230 polyclonal antibody (A) and anti-GFAP mAb (B). E18 rat cerebral astrocytes cultured on cover slips were fixed at day 5 *in vitro*, permeabilized, and incubated sequentially with anti-p230 polyclonal antibody and anti-GFAP mAb followed by rhodamine-conjugated horse anti-rabbit IgG and FITC conjugated goat anti-mouse IgG, respectively. p230-positive cells were also stained with anti-GFAP monoclonal antibody, indicating that p230 is produced by astrocytes. Bar = 100 μm.

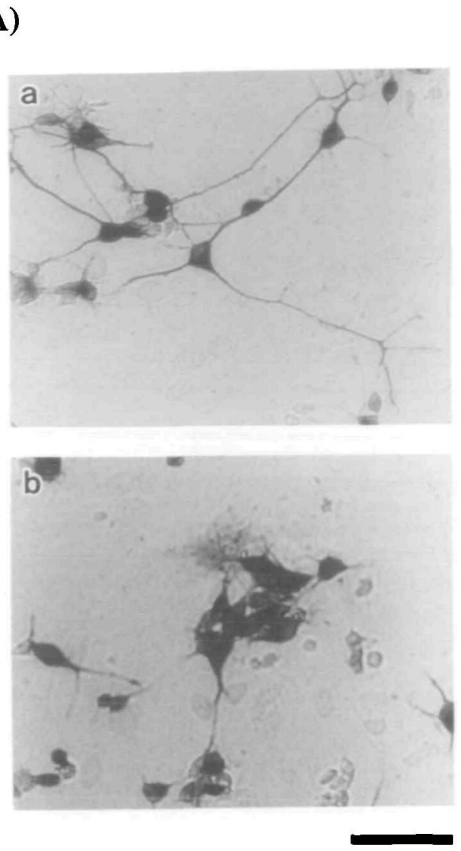

Fig. 5. **Inhibition of neurite outgrowth by anti-p230 polyclonal antibody.** (A) The effects of anti-p230 polyclonal antibody on neurite outgrowth of cerebral cortex neurons grown on the monolayer of astrocytes were examined. Freshly prepared cortical neurons were cultured for 48 h in the presence of either control IgG (a) or anti-p230 polyclonal antibody (b), then the cells were fixed and immunostained with anti-MAP 2 antibody. Neurite length in the presence of anti-p230 polyclonal antibody was significantly reduced compared to the control. Bar = 100 μm. (B) Cells with neurites longer than twice the cell diameter were counted and the percentages of such cells in the total numbers of cells were calculated. The results were presented as the mean ± SEM for 3 separate experiments. The percentage of cells was 51.2 ± 2.1% (solid bar) in anti-p230 antibody-treated cultures and 91.9 ± 3.4% (open bar) in nonimmune IgG-treated cultures (mean ± SEM, $n = 3$). Significance was attributed to $p < 0.05$.

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pig tenascin    812 DVTDTTALITWFK 824
                    *************
fragment 1          DVTDTTALITWFK

pig tenascin    887 ETFTTGLDAPRNLRRISQTDNSITLEWRNGK 917
                    ******************************
fragment 2          ETFTTGLDAPRNLRRISQTDNSITLEWRNGK

pig tenascin    998 ESSLTLLWRTPLAK 1011
                    ********* ****
fragment 3          ESSLTLLWRMPLAK

pig tenascin   1597 EEFWLGLDALSK 1608
                    ************
fragment 4          EEFWLGLDALSK

pig tenascin   1719 GHEYSIQFAEMK 1730
                    ************
fragment 5          GHEYSIQFAEMK
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Fig. 6. **Partial amino acid sequences of p230.** Partial amino acid sequences of p230 purified from fetal porcine brains were determined (fragments 1-5). Amino acid sequences of pig tenascin-C were derived from database search (accession number X61599) and compared with those of p230, with identical residues indicated by asterisks. Amino acids are shown by single letters, and the numbers from N-termini are indicated at the left.

The molecules which regulate neurite outgrowth should be expressed in a spatially restricted pattern, such that they are expressed only early in development. To investigate whether p230 fulfills this requirement, we analyzed the expression of p230 during development. Brain lysates from various developmental stages were analyzed in immunoblotting with anti-p230 polyclonal antibody (Fig. 3). A 230 kDa protein was detected in brain extracts from embryonic and postnatal rats but was hardly detectable in the adult, which is in good agreement with the observation in porcine brains. The intensity of the 230 kDa band was prominent in the embryonic and early postnatal stage, and thereafter decreased, indicating that the expression of the antigen may be temporally restricted in the rat brain.

To determine the cellular and subcellular distribution of p230, immunocytochemistry was performed on primary cultured neurons dissected from E18 rat cerebral cortex. Intense staining was observed in the cells other than neurons (data not shown), whose morphology was similar to that of astrocytes. To verify that the immunopositive cells were astrocytes, double immunolabeling with anti-p230 and anti-GFAP antibody was performed (Fig. 4). The immunoreactivity of p230 was detected on GFAP-positive cells, indicating that p230 is expressed on astrocytes.

Inhibition of Neurite Outgrowth by Anti-p230 Polyclonal Antibody—To investigate the physiological function of p230, cerebral cortex neurons were cultured on the monolayer of astrocytes in the presence of anti-p230 polyclonal antibody (Fig. 5). Cerebral cortex neurons grown on astrocyte monolayer exhibited an elaborate morphology, extending long and highly branched neurites. In contrast, neurite length in cultures treated with anti-p230 antibody was significantly reduced. In the control experiments, nonimmune IgGs and PBS were added to the culture, but they exhibited little effect on neurite outgrowth. The percentages of cells with neurites longer than twice the cell diameter were (mean ± SEM): anti-p230 antibody treated, 51.2 ± 2.1%, control, 91.9 ± 3.4%. This result suggests that p230 is required for neurite outgrowth from cortical neurons cultured on monolayer of astrocytes.

Partial Amino Acid Sequences of p230—Partial amino acid sequences of p230 purified from fetal porcine brains were determined. All determined sequences were highly homologous with porcine tenascin-C (Fig. 6), which is an extracellular matrix protein (*17*). The regions of tenascin

with homology to p230 are conserved through the tenascin family.

DISCUSSION

We report here the identification, isolation, and partial characterization of a cell surface protein, p230, that is expressed in fetal and neonatal brains. Double immunofluorescence analysis revealed that p230 is produced by GFAP-positive cells, indicating they are astrocytes. Our initial aim was to identify molecules specifically expressed on the growth cone membranes, although it is unlikely that p230 conforms to this criterion, since it is produced by astrocytes. Conceivably, p230 might rather be tightly associated and thereby co-fractionated with the growth cone membranes. This interaction between the growth cone membranes and p230 expressed on the surface of astrocytes is required for elongation of neurites, since neurite outgrowth from cerebral cortex neurons cultured on the monolayer of astrocytes was significantly reduced in the presence of anti-p230 polyclonal antibody. Collectively, these data led us to conclude that p230 is an extracellular matrix protein specifically produced by fetal and neonatal astrocytes, and plays a critical role in growth cone guidance.

On Western blot analysis using the anti-p230 mAb, p230 was detected only in the fetal brain fraction. However, the anti-p230 polyclonal antibody recognized an approximately 230 kDa protein in some other tissues from fetal pigs, indicating that isoforms of p230 are also expressed in tissues other than brain (data not shown). Therefore the monoclonal antibody might be limited to the brain isoform(s) of p230.

Partial amino acid sequence analysis demonstrated that p230 is highly homologous to an extracellular matrix protein, tenascin-C (Fig. 6). In addition, immunohistochemical analysis of coronal section of freshly frozen E18 rat forebrain revealed that the distribution of p230 immunoreactive regions, which covered the cortex and subcortical white matter (data not shown), was similar to that of tenascin-C (*18*). Indeed, p230 has some properties similar to tenascin-C: (i) p230 exhibits similar molecular weight on reducing SDS-PAGE; (ii) it is expressed by astrocytes but not by neurons; (iii) in brain, it is especially prominent in the embryonic and early postnatal stage, but not in the adult. These results strongly suggest that p230 is identical to, or a structural homologue of tenascin-C. In some respects, however, p230 has distinct characteristics from those previously reported for tenascin-C (see below).

Tenascin-C is a large multimeric protein consisting of repeated structural modules including heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III repeats, and a globular domain shared with the fibrinogens. Recently, it was found that these separate domains might mediate its diverse functions for neurons, such as cell adhesion, anti-adhesion, and promotion of neurite outgrowth (*19*). There have been many reports on the function of tenascin-C in neurite outgrowth, which is extremely variable depending on cell lineage. For example, both cell bodies and growth cones of CNS neurons are deflected from tenascin-C boundaries on patterned substrates consisting of components such as laminin-1 or poly-DL-ornithine which alternate with areas additionally containing the glycoprotein (*20, 21*). Moreover, retinal neurons do not grow neurites on a homogeneous tenascin-C containing surface (*22*). In contrast, tenascin-C promotes neurite outgrowth by E7-8 DRG neurons, E3 motor neurons, E18 hippocampal neurons and P6 cerebellar neurons (*21, 23, 24*). In these reports, all assays of cellular interaction with tenascin-C were performed on substrate-bound tenascin-C. We performed similar types of experiments with p230 and found that cerebral cortex neurons were able to grow on p230 adsorbed to PEI-coated dishes and exhibited no decrease or increase in neurite length when compared to the control (data not shown). In this regard, it is suggested that p230 is functionally distinct from tenascin-C, but conformational changes might abolish the neurite outgrowth inhibiting/promoting property of p230 when it is used as substrate. Alternatively, the neurite outgrowth inhibiting/promoting property of p230 might require the presence of other factor(s). For example, immunoaffinity purified N-CAM cannot exhibit homophilic binding in bead aggregation assay (*25*). N-CAM depends on heparin for its homophilic binding (*25, 26*). Therefore isolated N-CAM might function less because of losing heparin during isolation procedures.

We have found no reports concerning the function of tenascin-C in neurite outgrowth on the surface of astrocytes, although polyclonal antibody to tenascin-C had been found to reduce the binding of neurons to monolayers of astrocytes (*27, 28*). In contrast, anti-p230 polyclonal antibody did not appear to have any effect on the binding of cerebral cortex neurons to astrocytes in our assays. As discussed above, p230 produced by astrocytes might be tightly associated with growth cone membranes in the embryonic stage. Neurite outgrowth on the surface of astrocytes probably requires the interaction of the tips of neurites with p230.

We are grateful to Dr. N. Dohmae, Dr. M. Chijimatsu, Dr. K. Takio of the Institute of Physical and Chemical Research (RIKEN) for determination of amino acid sequences. We also thank Dr. S. Nakamura and N. Nakamuta for their assistance in preparation of brain sections.

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