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Identification and Characterization of Porcine NP-190, a Novel Protein That Is Specifically Expressed in the Axonal Membrane during the Embryonic Period[1]

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To identify and analyze the function of proteins expressed in the growth cones, we have screened monoclonal antibodies raised against the preparation of the growth cone particles derived from fetal porcine brains and found a novel neuronal antigen, termed NP-190. Biochemical characterization of NP-190 demonstrated that it was an integral membrane protein with an apparent molecular weight of 190 kDa and that it was mainly expressed in fetal brains. Homologous antigens with molecular weights of 200 and 170 kDa were also identified in the fetal brain extracts of chickens and rats, respectively. Immunoblot experiments of brain extracts from chickens and rats in various stages of development indicated that the expression of NP-190 homologs was developmentally regulated; it began to appear and increased in the embryonic stage, then decreased to very low level in the adult brains. Immunostaining of cultured primary of neurons from the embryonic day 18 rat cerebral cortex demonstrated that rat NP-190 homolog localized in the cell bodies, axons and growth cones, but not in dendrites. Partial amino acid sequence analysis of affinity-purified NP-190 from fetal porcine brains demonstrated that it was a novel protein. These results suggest that NP-190 plays a distinct role in brain development.

Key words: axonal pathfinding, brain development, cerebral cortex, growth cones, neurite growth.

During embryogenesis, the neuron undergoes dramatic changes in phenotype as it differentiates from a dividing neuroepithelial cell into a postmitotic, sprouting neuron and forms axons and dendrites. The leading edge of the neurite, the nerve growth cone, must follow an appropriate path to the target and synapse with its particular target cell. Generally, there are four possible types of signals that could guide axons toward their targets: short-range repulsive and attractive cues provided by molecules on nerve and glial cell surfaces, and diffusible chemoattractants and chemorepellents, which can act over longer distances.

Cell surface proteins, especially the cell adhesion molecules that were the first to come on the axonal guidance and outgrowth scene, are involved in both attraction (*1, 2*) and repulsion (*3*). The use of hybridoma technology and the production of monoclonal antibodies (mAbs) have often been crucial for the identification of cell surface antigens, which are involved in nervous system development of the leech (*4*), *Drosophila* (*5, 6*), and vertebrates (*7-11*). Intensive research efforts have been directed towards the identification of novel surface antigens in the hope of elucidating the precise molecular mechanisms which underlie cell adhesion (*12, 13*), axon pathfinding (*14*), and fasciculation (*15*).

Our experimental approach to the analysis of neurite growth-specific molecules is the immunochemical comparison of components expressed during specific stages of neuronal development. In our search for specific molecules whose expression is developmentally regulated in brains, we have produced hybridomas by fusion of SP2 myeloma cells with splenocytes immunized with purified growth cone particles (GCPs) from porcine brains. We have found a mAb which recognizes a developmentally regulated neuronal antigen, termed NP-190. Biochemical and immunological experiments have shown that NP-190 is a membrane protein and is present only during embryonic and neonatal stages. Immunostaining of cultured primary neurons with anti-NP-190 mAb has indicated that it is specifically localized to proximal axonal segments, cell bodies, and growth cones. Based on these results, we suggest that NP-190 is a novel protein expressed in early stages of neuronal development.

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Abbreviations: DAB, 3,3′-diaminobenzidine tetrahydrochloride; ELISA, enzyme-linked immunosorbent assay; GCPs, growth cone particles; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; i.p., intraperitoneal; i.v., intravenous; mAb, monoclonal antibody; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; r.t., room temperature; SS, synaptosome; TBS, Tris-buffered saline.

EXPERIMENTAL PROCEDURES

Animals—Fetal and adult porcine brains were purchased from the Shibaura Zoki. BALB/c mice and Wistar rats were purchased from Sankyo Labo Service. Fertilized eggs were from Nippon Biosupply Center.

Preparation of Growth Cone Particles (GCPs)—GCPs were isolated from fetal porcine brains as described by Ellis *et al.* (*16*) with slight modifications. Fetal porcine brains were homogenized in 8 volumes of 0.32 M sucrose with 5 mM HEPES and 0.5 mM EDTA, pH 7.4, containing a mixture of protease inhibitors (0.5 mM PMSF, 1 μg/ml each of antipain, and leupeptin). After centrifugation at 1,660 $\times g$ for 10 min, the supernatant was recovered, layered onto a discontinuous sucrose gradient (0.75 M; 1.0 M; 2.0 M) and was centrifuged at 100,000 $\times g$ for 60 min at 4°C. The 0.32 M sucrose (load)/0.75 M sucrose interface was recovered and pelleted at 39,800 $\times g$ for 30 min at 4°C. This GCP pellet was resuspended for use as described.

Preparation of Synaptosome (SS)—SS was prepared from adult porcine brains essentially according to the procedure of Cohen *et al.* (*17*) with modifications. Briefly, adult porcine brains were homogenized in 8 volumes of 0.32 M sucrose with 5 mM HEPES and 0.5 mM EDTA, pH 7.4, containing protease inhibitors. After centrifugation at 750 $\times g$ for 10 min, the supernatant was centrifuged at 17,300 $\times g$ 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 $\times g$ 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 $\times g$. The pelleted SS fraction was resuspended in phosphate-buffered saline (PBS).

Generation of Monoclonal Antibodies (mAbs)—BALB/c mice received an initial intraperitoneal (i.p.) injection of pelleted GCPs (100 μg) in PBS, mixed 1:1 with Freund's complete adjuvant. Over a period of months, mice were further immunized with i.p. injections of GCPs plus Freund's complete adjuvant, then given a final intravenous (i.v.) injection of GCPs in PBS. Four days after the i.v. injection, the spleen was aseptically removed from the immunized mice, and splenocytes were prepared. Splenocytes were fused with SP2 myeloma cell line using polyethylene glycol-6000. Cells were fused at a ratio of 10 splenocytes to 1 myeloma and plated into 96-well plates in Dulbecco's modified Eagles medium containing HAT supplements. The cells were maintained at 37°C in air with 10% CO_2 added. Hybridomas were then switched to HT medium (HAT medium minus the aminopterin) on day 14 after the fusion. Wells containing >50% confluent hybridoma growth were assayed for antibody production.

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

Preparation of Tissue Lysates—Crude cell extracts used for antigen identification by immunoblotting were obtained as follows: porcine tissues were removed and homogenized in 0.32 M sucrose with 5 mM HEPES and 0.5 mM EDTA, pH 7.4, containing protease inhibitors in a Dounce homogenizer. The homogenates were centrifuged for 15 min at 1,500 $\times g$ at 4°C, the pellet was discarded, and the supernatant was analyzed by SDS-PAGE and immunoblotting. Chicken and rat brains of different embryonic (E) and postnatal (P) ages (in days) were similarly treated. Protein estimations were performed according to Lowry *et al.* (*18*) using bovine serum albumin as a standard.

Extraction of NP-190 from the Membrane Fractions—Crude cell lysate was prepared by homogenizing from fetal porcine brains in 5 mM HEPES containing 0.32 M sucrose and 0.5 mM EDTA, pH 7.4. This was centrifuged at 1,500 $\times g$ for 15 min, and the supernatant was separated into a soluble fraction and a membrane pellet by ultracentrifugation at 100,000 $\times g$ for 1 h. The membrane pellet was resuspended and incubated in 1 M NaCl, 0.1 M Na_2CO_3 (pH 11) or 1% Triton X-100 for 40 min at 4°C, followed by ultracentrifugation at 100,000 $\times g$ for 1 h. The resulting pellet was resuspended in TBS in the same volume as supernatant. These pellets and supernatants were analyzed by Western blotting using NP-190 mAb as a probe.

Gel Electrophoresis and Immunoblotting—SDS-PAGE was performed on gels containing 7.5% acrylamide according to Laemmli (*19*). Protein samples separated by SDS-PAGE were transferred to nitrocellulose (Schleicher & Schuell BA85, 0.45 μm) according to the standard procedures (*20*). The nitrocellulose was blocked for 30 min with 5% skim milk in TBS and incubated with the NP-190 mAb (hybridoma culture supernatant or purified IgG fraction from ascites fluid in 1:1,000 dilution) for 1 h at r.t. After washing in TTBS (TBS containing 0.05% Tween 20), the nitrocellulose was incubated with horseradish peroxidase-conjugated anti-mouse IgG. The nitrocellulose was developed in 4-chloro-1-naphthol or 3,3′-diaminobenzidine tetrahydrochloride (DAB) with nickel enhancement.

Immunoaffinity Purification of NP-190—Immunoaffinity chromatography was performed as described by Schneider *et al.* (*21*). Briefly, NP-190 mAb, purified from ascites using MAbTrap G II, was coupled to protein G-Sepharose CL-4B (15 mg/3 ml packed beads; Pharmacia). Antibody was then cross-linked to the beads using dimethyl pimelimidate. A homogenate was prepared from fetal porcine brain in 5 mM HEPES (pH 7.4) containing 0.32 M sucrose, 0.5 mM EDTA, and a mixture of protease inhibitors. This was centrifuged for 15 min at 1,500 $\times g$ at 4°C, the pellet was discarded and the supernatant was centrifuged at 100,000 $\times g$ for 1 h. The pellet was resuspended for 30 min in TBS containing 0.5% Nonidet P-40 (NP-40), then centrifuged at 100,000 $\times g$ for 1 h again. The supernatant, a crude membrane fraction, was applied to the antibody-coupled column. Unbound protein was 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 and (2) 0.15 M NaCl, 0.05 M

Tris-HCl (pH 8.2), 0.5% NP-40, 0.1% SDS. The NP-190 was eluted with 0.1 M diethylamine (pH 11), 0.1% SDS and 0.5% NP-40. The eluted sample was neutralized immediately with 1/10 volume of 1 M HEPES (pH 5).

Cell Culture—Cerebral cortex from 18-day gestation fetal rats was dissected, cut into small pieces and digested in papain solution for 10 min at 37°C. Then DNase I was added and incubated for 10 min at 37°C. The dissociated cells were collected and plated onto 96-well polyethyleneimine-coated plastic plates. The cells were grown at 37°C in Dulbecco's modified Eagles medium (Gibco #430-2100) containing insulin and transferrin (5μg/ml each) and 5% each of fetal calf serum and horse serum, in an atmosphere of humidified air containing 10% CO_2. Cultures were maintained for 3 to 7 days.

Immunostaining—Staining of cell cultures was performed by the immunoperoxidase method as described by Thomaidou and Patsuavoudi (*11*). Cultured neurons were fixed in 4% paraformaldehyde in PBS and washed in TBS. After fixation, cells were treated with 0.3% Triton X-100 for 3 min. Nonspecific binding sites were blocked with 5% skim milk in TBS for 30 min. Cells were incubated with the primary antibody diluted in TBS for 1 h, and the secondary antibody (anti-mouse IgG conjugated to horseradish peroxidase) for 1 h at r.t. Immunoreactivity was visualized using DAB as a substrate with nickel enhancement.

Amino Acid Sequence Analysis of NP-190—Immunoaffinity-purified NP-190 from the fetal porcine brain was separated by SDS-PAGE, and the protein band of NP-190 visualized by Coomassie staining was cut out. The protein was eluted from the gel into 5 ml of SDS-PAGE electrophoresis buffer for 3 h at 200 V according to the method of Kobayashi *et al.* (*22*). The eluted protein (*ca.* 20 μg) was concentrated, mixed with V8 protease (0.1 μg) and immediately run on SDS-PAGE (10% gel). When the front marker reached the separation gel, the electrophoresis was stopped for 15 min, then restarted. The digested peptide fragments were transferred to the PVDF membrane according to Matsudaira (*23*) and the separated peptides were visualized with Coomassie blue. A portion of the membrane was stained with anti-NP-190 mAb and the protein band which reacted with the antibody was recovered. The amino acid sequence was determined by ABI model 473A protein sequencer.

RESULTS

Production of mAb against NP-190—To elucidate the functional and structural components of growth cones, we attempted to isolate and characterize a fraction that is enriched with growth cones from porcine brains. To this end, the method for isolation of growth cones described by Pfenninger *et al.* (*16, 24*) was employed. Fetal porcine brains were homogenized, separated by discontinuous sucrose density gradients, and growth cone particles (GCPs) fraction was isolated as described in the "EXPERIMENTAL PROCEDURES." To test whether the GCPs fraction we had isolated was enriched with authentic growth cones, we performed Western blot analysis using the mAb against GAP-43, which had been shown to be enriched in growth cones by immunoblot (*25*) and immunofluorescence (*26*) analyses. GAP-43 was enriched in our preparation of GCPs compared with other fractions of the purification procedure (data not shown), in almost the same way as previously reported (*27*). This GCP fraction was used as antigen to immunize 8-week-old BALB/c mice to generate monoclonal antibodies against developmentally regulated antigens of growth cones. The fusion of myeloma cells with splenocytes from immunized mice resulted in ~400 hybridomas. Each hybridoma supernatant was screened differentially against GCPs *versus* synaptosome (SS) proteins as antigens by an enzyme-linked immunosorbent assay (ELISA). Hybridomas producing antibodies with higher reactivity against GCPs than SS were selected. This selection procedure was repeated three times, and approximately 80 positive clones were selected. One of these, which produced the mAb against a 190-kDa protein, was chosen for further study. Since this protein was later shown to be a novel brain-specific protein, we named it NP-190. The hybridoma was re-cloned twice by the method of limiting dilution. Anti-NP-190 mAb was later shown to belong to the IgG subclass.

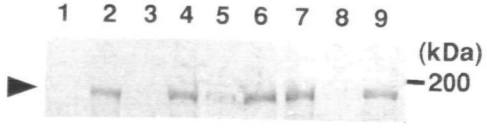

Fig. 1. **NP-190 is an integral membrane protein.** Fetal porcine brain extract (lane 9) was separated by ultracentrifugation into soluble (lane 1) and pelleted (lane 2) fractions. The pellet was further extracted with 1 M NaCl (lanes 3 and 4), 0.1 M Na_2CO_3 (lanes 5 and 6), or 1% Triton X-100 (lanes 7 and 8), followed by second ultracentrifugation for separation of supernatants (lanes 3, 5, and 7) and pellets (lanes 4, 6, and 8). All the samples were analyzed by SDS-PAGE and immunoblotting with anti-NP-190 mAb. The location of NP-190 is indicated by an arrowhead.

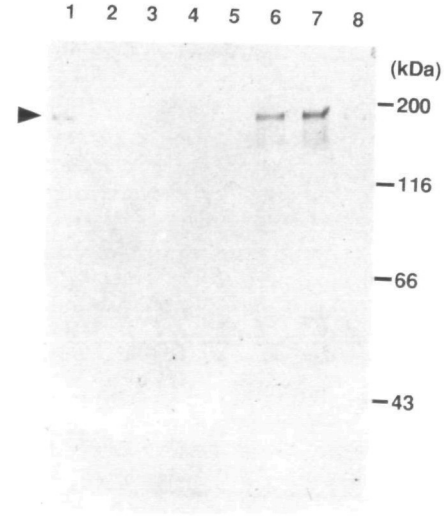

Fig. 2. **Expression of NP-190 in various porcine tissues.** Total cell extracts from fetal porcine tissues (lane 1, skeletal muscle; lane 2, kidney; lane 3, spleen; lane 4, liver; lane 5, lung; lane 6, heart; lane 7, brain), or adult porcine brain extracts (lane 8) were prepared. Proteins (6 μg) of each sample were resolved on 7.5% SDS-PAGE, transferred to nitrocellulose and probed with anti-NP-190 mAb. The expression of NP-190 was most abundantly observed in the fetal brain, but lower level of expression was also detected in fetal heart and skeletal muscle fractions (arrowhead). The positions of the molecular weight markers (in kDa) are indicated at the right.

NP-190 Is an Integral Membrane Protein Specifically Expressed in Fetal Brains—NP-190 was detected in the pellet derived from the 100,000 × *g* centrifugation (Fig. 1, lane 2), suggesting that it was associated with the membranes. To further investigate the nature of association of NP-190 with the membranes, cell extracts were incubated in buffer containing 1 M NaCl, 0.1 M Na_2CO_3 (pH 11), or 1% Triton X-100. Following centrifugation at 100,000 × *g* the resulting supernatant and pelleted fractions were analyzed by immunoblotting. As shown in Fig. 1, after the extraction with 1 M NaCl or 0.1 M Na_2CO_3, NP-190 appeared in the pelleted fractions (lanes 4 and 6), but it was solubilized by extraction with Triton X-100 (lane 7). This result suggested that NP-190 was an integral membrane protein.

To examine the expression of NP-190 in tissues other than brain, equal amounts of crude cell extracts from fetal skeletal muscle, kidney, spleen, liver, lung, heart, and brain along with adult brain were submitted to an immunoblotting experiments. Figure 2 demonstrates that NP-190 is most abundant in fetal brain extract (lane 7), although it can also be detected in adult brain (lane 8), fetal heart (lane 6), and fetal skeletal muscle (lane 1) preparations. Collectively, these results demonstrate that NP-190 is an integral membrane protein mainly expressed in fetal brains.

Preparation of Polyclonal Antibody against NP-190 and the Expression of NP-190 Homolog during Brain Development in Chickens and Rats—Since the preliminary experiments using fetal and adult porcine brain extracts indicated that the expression of NP-190 was confined to the fetal stage, it was of interest to examine more closely the changes of the amount of NP-190 during brain development. However, mAb against NP-190 reacted only with brain extract from pigs, but not with those from other animal species such as chickens, rats and mice (data not shown). Therefore, we purified NP-190 from fetal porcine brain extract to obtain the polyclonal antibody. To this end, an immunoaffinity column was prepared by immobilizing anti-NP-190 mAb from ascites fluid onto protein G Sepharose CL-4B column. Enriched membrane extract obtained from fetal porcine brains was solubilized by treatment with 0.5% NP-40 and passed over the affinity column. After washing the column several times, NP-190 was eluted by alkaline pH and was analyzed by SDS-PAGE (Fig. 3). Because NP-190 decomposed easily, lower molecular weight bands were detected. Heavy and light chains of anti-NP-190 mAb were also present in the alkaline eluate, possibly due to release from the solid-state carrier under alkaline elution conditions. The main band of NP-190 was cut from gel and used for immunization in mice.

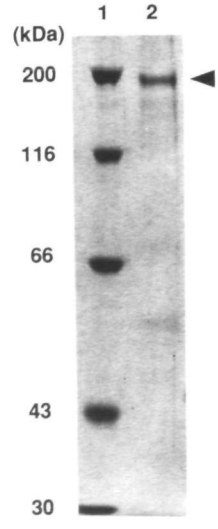

Fig. 3. **Immunoaffinity purification of NP-190 from fetal porcine brains.** NP-190 was purified from fetal porcine brains by immunoaffinity chromatography using anti-NP-190 mAb. A crude membrane fraction prepared from fetal porcine brains was passed through the affinity column coupled with anti-NP-190 mAb. The column was washed, and the proteins bound to the column were eluted with 0.1 M diethylamine (pH 11), neutralized and analyzed by SDS-PAGE (lane 2). The gel was stained with Coomassie Blue. The position of NP-190 is indicated by an arrowhead. Molecular weight markers (lane 1; from the top): 200, 116, 66, 43, and 30 kDa.

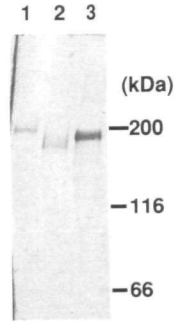

Fig. 4. **Immunoblot analysis of fetal brain extracts from chickens, rats, and pigs with anti-NP-190 polyclonal antibody.** Total extracts of fetal brains (6 μg) were prepared from chickens (lane 1; embryonic day 18), rats (lane 2; embryonic day 18), and pigs (lane 3) as described in "EXPERIMENTAL PROCEDURES." They were subjected to immunoblot experiments probed with anti-NP-190 polyclonal antibody. Proteins of apparent molecular weights of 200 and 170 kDa were detected in the fetal brain extracts of chickens and rats, respectively, along with 190 kDa NP-190 in the fetal porcine brain extracts. The locations of the molecular weight markers are indicated at the right.

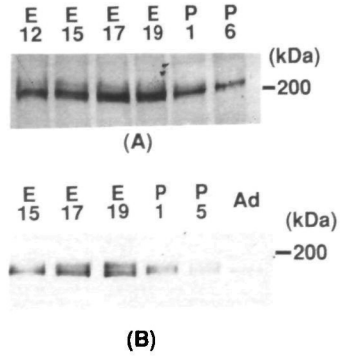

Fig. 5. **Expression of NP-190 homolog in the chickens and the rats during brain development.** Homogenates of brains at various stages of development from chickens (A) and rats (B) were prepared as described in "EXPERIMENTAL PROCEDURES" and analyzed by immunoblotting using the anti-NP-190 polyclonal antibody (1:250). The NP-190 homologs in chickens and rats became detectable as early as embryonic day (E) 12 and 15, with a maximum level of expression between embryonic day 17 (E17) and E19, and then began to decrease. E, embryonic day; P, postnatal day; Ad, adult brain. The locations of the molecular weight markers are indicated at the right.

The polyclonal antibody thus obtained was tested in immunoblotting experiments (Fig. 4). With this NP-190 polyclonal antibody, cross-reactive determinants were found in brain extracts from fetuses of chickens, rats, and pigs; polypeptides with apparent molecular weights of 200, 170, and 190 kDa were detected in fetal brain extracts from chickens, rats, and pigs, respectively.

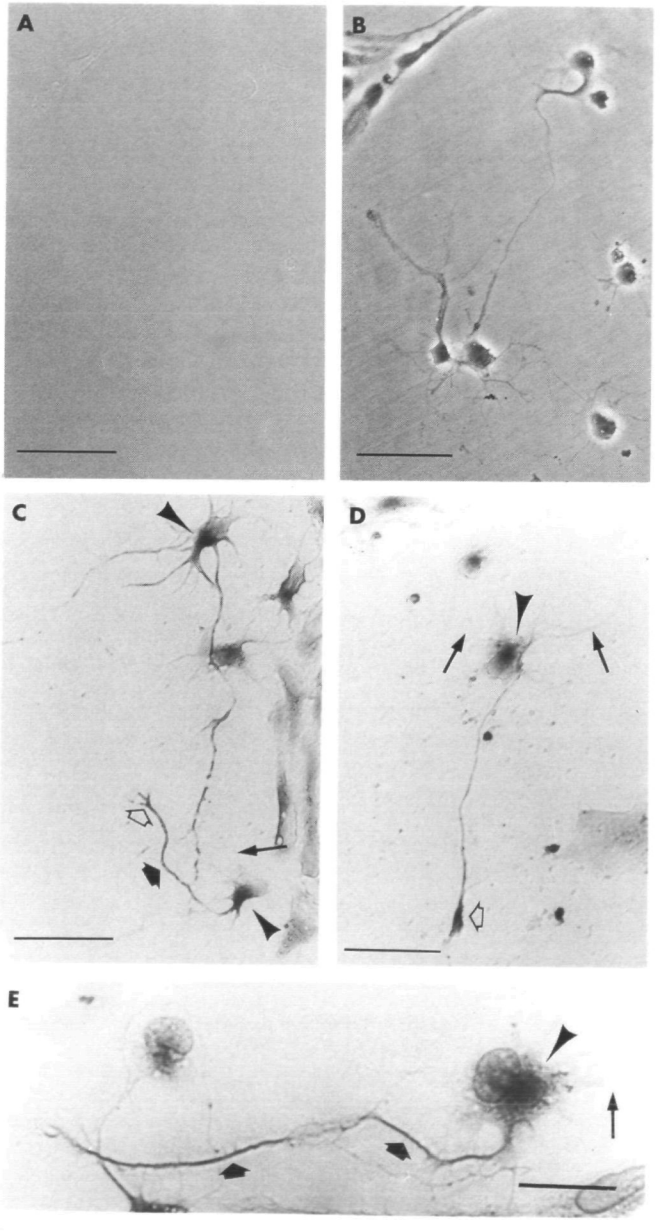

Fig. 6. **Indirect immunostaining with anti-NP-190 polyclonal antibody of dissociated rat cortical neurons grown *in vitro*.** Cortical neurons from fetal rat brains at embryonic day 18 were isolated and cultured as described in "EXPERIMENTAL PROCEDURES." The cultures were fixed and stained either without the addition of a primary antibody (A) or with mouse polyclonal antibody against NP-190 (C, D, and E), followed by incubation with horseradish peroxidase-conjugated anti-mouse-IgG. B, phase-contrast image. Note that strong immunostaining was seen in the cell bodies (arrowheads) and axon (thick arrows), but the dendrites were not stained (thin arrows). Growth cones were stained in some, but not all neurons (open arrows). Scale bar: A, B, C, and D = 100 μm. E = 50 μm.

This anti-NP-190 polyclonal antibody was then used to examine the expression of NP-190 homolog during chicken and rat brain development (Fig. 5). Cell extracts from chicken brains at days 12, 15, 17, and 19 of gestation (E12, E15, E17, and E19) as well as postnatal day 1 and 6 (P1 and P6) were blotted onto nitrocellulose and reacted as described. NP-190 homolog was detectable in cell extracts prepared from chicken brain on E12 and it increased toward E17, when it reached the maximum amounts (Fig. 5A). During the postnatal period, it decreased gradually; the antigen persisted at P6, but in diminished quantity. The expression of NP-190 homolog during development in rats was similarly tested, with essentially the same results (Fig. 5B). NP-190 homolog in rats became detectable with a maximum of expression between E17 and E19, and then began to decrease; it was hardly detectable in the adult brain preparations. These results suggest that NP-190 homologs are specifically expressed only during the embryonic and neonatal period.

Subcellular Distribution of Rat NP-190 Homolog in Cultured Neurons—The subcellular distribution of rat NP-190 homolog in neuronal cells was studied by an indirect immunoperoxidase method using dissociated cells from fetal rat cerebral cortex grown in culture for several days. The patterns of immunolabeling of paraformaldehyde-fixed neurons, permeabilized with Triton X-100, are shown in Fig. 6. No reaction can be observed in controls reacted without primary antibody (Fig. 6A). Specific immunostaining by anti-NP-190 polyclonal antibody was seen in the cell body, especially the proximal axonal segment, and the axon (Fig. 6, C, D, and E). Growth cones were stained in some neurons (Fig. 6, C and D), which is consistent with the fact that NP-190 was isolated from growth cone-enriched fractions. However, the dendrites were scarcely stained. These lines of evidence indicate that NP-190 is an axonal and growth cone membrane protein.

Amino Acid Sequence Analysis of NP-190—We determined the partial amino acid sequences of affinity-purified, V8 protease-digested NP-190 fragments (*28*). The N-terminal amino acid sequences of peptide fragments of 17, 25, and 23 kDa were analyzed, since these three fragments reacted with anti-NP-190 mAb. Their sequences were as follows: VAHDRKFKNVQPKD??KVP for the 17-kDa fragment, AIAVANPKEEVIVKNKDEK for the 25-kDa fragment and MADLRNVPK?DVI?K for the 23-kDa fragment. These sequences did not match any of the amino acid sequences in the SwissProt/PIR databases.

DISCUSSION

The nerve growth cones are involved in determining the direction and extent of neurite elongation during the nervous system development. They are highly mobile in structure, extending or retracting filopodial processes depending on the nature of the substrata they encounter. To date, several membrane proteins have been found to localize specifically on growth cones: the 5B4 antigen (*29*), growth-associated protein GAP-43 (*25*), c-*src* protooncogene product (*30*), and the synaptic vesicle associated protein synapsin I (*31*). None of these components, however, appears to be unique to the nerve growth cones, so the biochemical marker specific for the growth cones remains unknown.

In an attempt to characterize the molecular nature and function of the growth cones, we searched for growth cone- or nerve sprouting-specific marker proteins by screening monoclonal antibodies raised against growth cone fractions. We used fetal porcine brains as the source for isolation of the growth cones for the following reasons: (1) Fetal porcine brains are far larger than those of the other obtainable experimental animal species (*e.g.*, the weight of an E17 rat brain is around 0.1 g, while that of a fetal porcine brain is more than 20 g). This might increase the possibility that the antibodies against rare antigens could be obtained, since the recovery of proteins after the procedure of isolation of growth cones is relatively low. (2) Phylogenetically, pigs are far different from mice, so it is expected that antibodies against proteins with crucial functions might easily be generated in mice.

One of the monoclonal antibodies produced from mice immunized with growth cone particles (GCPs) recognized a 190-kDa protein, NP-190, which was present in the fetal brain in much greater amounts (on a protein basis) than the mature brain. NP-190 remained associated with the membrane fraction in high salt or alkaline pH conditions, while it was solubilized with Triton X-100, suggesting that it is an integral membrane protein (Fig. 1).

The NP-190 homolog was present in the chicken brain as early as E12 (Fig. 5). The data suggest that the NP-190 homolog may also be involved in the proliferative process. In the fetal brain, antigenicity peaks appear just before birth, when axonal growth is extensive and many synapses have yet to be formed. The expression of NP-190 homolog was not restricted to the growth cone; immunostaining analysis of isolated growing neurons has shown that NP-190 is also expressed in the cell body and axons, especially in the proximal region, but not in dendrites (Fig. 6). These lines of evidence suggest that NP-190 is involved in neurite formation or axonal guidance. Persistence of the NP-190 expression in the adult brain (at very low levels) does not conflict with our suggestion concerning its role during development, since upon reaching maturity it is certain that developmental processes may continue to operate at a lower level, so as to mediate neuronal plasticity.

An interesting feature of the NP-190 mAb is its tissue distribution. The NP-190 mAb failed to react with crude cell extracts prepared from fetal porcine kidney, spleen, liver, and lung, but it did react with heart and muscle proteins (Fig. 2). Recent research on the molecules involved in the formation of synapses has focused on the peripheral synapse between a motor neuron and a muscle cell, called the neuromuscular junction. Local specialization of pre- and post-synaptic cells is independent of synaptic activity and instead requires inductive signals. Agrin (*32, 33*), laminin $\beta 2$ (*34, 35*), and ARIA (*36*) are the best characterized proteins known to be involved in particular aspects of synaptic differentiation. Because the NP-190 exists simultaneously in both nervous and muscular systems, it may play an important role in neuromuscular development.

We have determined the partial amino acid sequences of affinity-purified, V8 protease-digested NP-190 fragments. These sequences were compared with sequences in the SwissProt/PIR databases and found to be novel. To elucidate the whole primary structure of NP-190, we have screened fetal porcine brain cDNA library constructed on λgt11 with monoclonal and polyclonal antibodies against NP-190 and have cloned partial cDNA of NP-190. Sequences encoding the peptide fragments of 17 and 25 kDa were found within the cDNA sequences of the isolated clones (data not shown). DNA sequence analysis of these isolated clones further confirmed that the NP-190 is a novel protein; no significant homology in DNA or protein sequences was found by searching the databases. Further molecular and biochemical analyses are needed to understand the function of NP-190 in brain development.

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