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Identification and Purification of a Chicken Brain Neuroglia-associated Protein*

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The identification, purification, and biochemical characterization of specific markers for neuroglial cells in the central nervous system is an essential step toward a better understanding of the function of glial cells. This manuscript reports the identification and purification of a neuroglia-associated protein (NAP-185) with an apparent molecular mass of 185 kDa. While its expression is not restricted to the brain, it was first identified in a specific subpopulation of glial cells when chick brain stem sections were analyzed with an affinity-purified rabbit antiserum raised against the catalytic domain of the T-cell protein tyrosine phosphatase. This 185-kDa antigen was purified to apparent homogeneity and confirmed to be responsible for the neuroglial staining observed. In spite of its immunological relation to T-cell protein tyrosine phosphatase, purified NAP-185 failed to display tyrosine phosphatase activity. The primary sequence of five NAP-185-derived peptides shows that this protein has not yet been characterized and that it is possibly related to AP180, a clathrin-associated protein.

Neuroglial cells represent 80–90% of the brain cells and have long been considered to have purely architectural functions, providing support and insulation to neurons and preventing electrical signals from spreading between nerve cells. Increased interest in these cells emerged when it was discovered that they actively participate in brain homeostasis (1) and in the transmission of nerve impulses (2, 3). Based on histological criteria, glial cells have been subdivided into two classes: macroglia (astrocytes, oligodendrocytes, and ependymal cells) and microglia (microcytes).

This morphological classification is to some extent supported by functional criteria, since defined physiological properties are apparently associated with a distinct glial cell type. For instance, oligodendrocytes synthesize and maintain the myelin layer that surrounds the axons enabling neurons to transmit electrical signals much faster. Ependymal cells synthesize components of the cerebrospinal fluid and promote its circulation with their cilia. Microcytes are involved in immunological reactions of the central nervous system. They up-regulate many of their immune-related antigens upon activation (4) and can function as antigen-presenting cells (5).

The exact roles of astrocytes still remain elusive. These cells are generally defined at the light microscope level by the presence of glial fibrillary acidic protein, an intermediate filament protein. They can appear in a variety of morphologies depending on their location, and glial fibrillary acidic protein-positive cells have been implicated in several aspects of brain function (6). It is speculated that astrocytes modulate neuronal activity by sensing and responding to neuronal signals because of their ability to express a diversity of ligand-gated and voltage-gated channels (2). In addition, astrocytes are known to be physically associated with synapses (1) and nodes of Ranvier (7), both of which are highly specialized structures involved in the transmission of nerve impulse. *In vivo* and *in vitro* (8) studies indicate that astrocytes play an important role in determining the properties of the endothelial cells that form the blood-brain barrier. Therefore, they contribute to the development of the "immune privileged" status of the brain. The ability of some astrocytes to express major histocompatibility complex II antigens suggests that they can act as antigen-presenting cells and may be important in the etiology of autoimmune diseases such as encephalomyelitis (9, 10).

The most remarkable characteristic of astrocytes is their ability to undergo hyperplastic and hypertrophic transformation in response to neuronal degeneration, a phenomenon known as astrogliosis. These "reactive astrocytes" are thought to favor the repair process and are essential to neuronal survival. Indeed, reactive astrocytes may be substrates for regenerating axons (11, 12), and they can synthesize and release neurotrophic factors (13, 14).

We have previously shown that deafferentation of the nucleus magnocellularis of the chick brain stem induces astrogliosis (15). The transition of astrocytes from the resting to the reactive state requires proliferative pathways to be turned on and most likely involves tyrosine phosphorylation/dephosphorylation events. In order to determine whether protein tyrosine phosphatases play a role in the activation process leading to astrocytosis, control and deafferented chick brain stem sections were screened with antisera specific for selected protein tyrosine phosphatases. An antiserum raised against the catalytic domain of T-cell protein tyrosine phosphatase (16) labeled a distinct population of neuroglial cells. Surprisingly, this antiserum did not immunoprecipitate the 48-kDa T-cell protein tyrosine phosphatase in chick brain extracts, but rather it immunoprecipitated a 185-kDa protein. Because it was first identified in neuroglial cells it was named neuroglia-associated protein 185 (NAP-185).[1] This manuscript reports the identification and purification of NAP-185, a new astroglial marker.

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[1] The abbreviations used are: NAP-185, neuroglia-associated protein 185; AP180, clathrin-associated protein 180; mAb, monoclonal antibody; 5501 Ab, affinity-purified 5501 antibody; 2844 Ab, affinity-purified 2844 antibody; BrdU, bromodeoxyuridine; MES, 4-morpholineethanesulfonic acid; MAP, microtubule-associated protein.

EXPERIMENTAL PROCEDURES

Materials

DE52 was purchased from Whatman. S Sepharose Fast Flow, Superose 12, and Sepharose 4B were from Pharmacia Biotech Inc. α-Casein was purchased from Nutritional Biochemicals Corp. and coupled to CNBr-activated Sepharose 4B. Protein A-Sepharose and Sepharose 4B were from Sigma. Fresh chicken heads were provided by ACME Poultry Co. Inc. (Seattle, WA).

Buffers

All percentages are given in (v/v) except where indicated. Buffer A contained 10 mM imidazole HCl (pH 7.2), 4 mM EGTA, 4 mM EGTA, 5% glycerol, 0.1% β-mercaptoethanol, 0.002% (w/v) phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 μg/ml leupeptin, 0.5 μg/ml pepstatin A, and 20 KIU/ml aprotinin. Buffer B contained 10 mM imidazole HCl (pH 7.2), 2 mM EDTA, 2 mM EGTA, 5% glycerol, 0.1% β-mercaptoethanol, 0.002% (w/v) phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.1% Triton X-100. Buffer C contained 10 mM MES (pH 6.0), 2 mM EDTA, 2 mM EGTA, 5% glycerol, 0.1% β-mercaptoethanol, 0.002% (w/v) phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.1% Triton X-100.

Antisera

Two polyclonal antisera were raised in New Zealand white rabbits. Rabbit 5501 was immunized with the catalytic domain of T-cell protein tyrosine phosphatase (16), and peptide B coupled to keyhole limpet hemocyanin was used to immunize rabbit 2844. Both antisera were affinity purified and are further referred to as 5501 Ab and 2844 Ab respectively. Mouse monoclonal antibody (mAb) AP180-I was a generous gift from Dr. Ungewickell (Washington University, St-Louis). The monoclonal cell supernatant was used at a dilution of 1:4, and polyclonal antisera were used at 1:1000 unless otherwise stated.

Immunoprecipitations and Western Blot Analysis

Tissue samples were minced on ice and homogenized in 5 volumes of Buffer A with a Polytron (Kinematica, Luzern, CH) using two 15-s bursts at full speed. Homogenates were adjusted to a 1% final concentration with Triton X-100 and maintained on ice for 30 min with occasional vortexing. The samples were then clarified by centrifugation at 16,000 × *g* for 15 min at 4 °C. The protein concentration of the extracts was routinely adjusted to 1 mg/ml. Aliquots of 1 ml were incubated at 4 °C overnight with 5 μl of antiserum and 10 μl of packed protein A-Sepharose. The beads were then washed 5 times with 25 mM HEPES pH 7.2, 150 mM NaCl, 10% glycerol, 0.1% β-mercaptoethanol, and 0.1% Triton X-100. The processed samples were resuspended in Laemmli sample buffer (17).

Subjects

Posthatch white Leghorn chickens (7–14 days old) were used for immunocytochemistry experiments. The eggs were obtained from H&N Farms, Redmond, WA, and maintained in the University of Washington facilities.

Tissue Preparation for Histochemistry

Chicks were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg/kg) and transcardially perfused with chick Ringer's (154 mM NaCl, 6 mM KCl, 8.4 mM $MgCl_2$, 5 mM HEPES, 8 mM glucose, and 1 mM EGTA) for 3 min, and the brains were removed and postfixed in a modified Carnoy's fix (ethanol, chloroform, glacial acetic acid, 10 × Ringer's) (6:2:1:1, v/v/v/v) at 4 °C for 6 h. The brains were then rinsed with 70% ethanol, left in 70% ethanol overnight, and embedded in paraffin the following day. Ten-micron sections of the brain stem were prepared, mounted onto chrome alum subbed slides, and deparaffinized.

The animals (n = 5) used in double labeling experiments were subjected to a unilateral cochlea removal as described previously (18). After 48 h, they received a subcutaneous injection of bromodeoxyuridine (BrdU) (50 mg/kg). Six hours later the animals were processed as described above for control animals (n = 3).

Immunocytochemistry

Unless noted otherwise, immunocytochemical reagents were prepared in Buffer H (100 mM Tris-HCl, pH 7.4, 1% bovine serum albumin, and 0.1% NaN_3).

Double-labeling Immunocytochemistry—Tissue slides obtained from BrdU-injected animals were deparaffinized and sequentially immersed in water (10 min), 1 N HCl (20 min) and washed in 100 mM Tris-HCl (pH 7.4). Blocking was achieved with 4% horse serum for 20 min. They were further incubated with an anti-BrdU mAb (Becton Dickinson, San Jose, CA; 1:300), overnight at room temperature in a humidified atmosphere. The sections were then stained as described below. Staining was enhanced by the addition of 0.08% $NiCl_2$ to the chromogen solution.

Routine Immunochemistry—Control sections were deparaffinized and blocked for 20 min in 4% goat serum. All tissue sections were incubated overnight at 4 °C with either 5501 Ab (1:3000) or 2844 Ab (1:6000) in a humidified chamber. The slides were then washed in Buffer H, incubated for 1 h with biotinylated horse anti-mouse antibody at a dilution of 1:250 (Vector Labs, Burlingame, CA), washed in 100 mM Tris-HCl (pH 7.4), and finally incubated in Vectastain ABC reagent (Elite kit, Vector Labs) prepared in Buffer H containing no NaN_3. Staining was performed for 10 min using 0.25 mg/ml diaminobenzidine (Sigma) and 0.1% H_2O_2 in 100 mM Tris-HCl, pH 7.4. The reaction was stopped by rinsing the samples twice with water, once with 100 mM Tris-HCl (pH 7.4), and once with Buffer H. The samples were dehydrated with ethanol and coverslipped with DPX mountant (BDH Chemicals Ltd. Poole, UK).

Purification of NAP-185

Centrifugation and chromatographies were carried out at 4 °C. All other procedures were performed at 0 °C.

Step 1: Extraction—Fresh chicken heads were kept on ice and rapidly processed. The brains were dissected out and washed extensively with water to remove blood clots. The tissue (300 g) was homogenized in a Waring blender for 4 periods of 15 s each using 3 volumes of Buffer A. The homogenate was adjusted to a final concentration of 2% Triton X-100, stirred continuously for 30 min, and centrifuged at 27,000 × *g* for 30 min. The soft pellet was discarded, and the supernatant was gently filtered through a coarse fritted glass filter and adjusted to 6 liters with Buffer A.

Step 2: Batch Chromatography on DE52—Packed DE52 cellulose (30 ml/g of protein) was equilibrated in Buffer A, added to the 6-liter extract and gently stirred for 6 h. The slurry was drained under mild suction using a 2-liter Pyrex buchner funnel with a coarse fritted disc, and the DE52 pad was washed with 2 liters of Buffer B. The DE52 cellulose was packed in a 5 × 38 cm column and developed with a linear gradient from 0 to 0.4 M NaCl in a total volume of 3.6 liters of Buffer B at a flow rate of 7.5 ml/min. A total of 170 fractions (18 ml each) were collected and subjected to Western blot analysis using antiserum 5501.

Step 3: Chromatography on α-Casein-Agarose—The immunoreactive fractions (60–90) were pooled, dialyzed against Buffer B, and applied to a 2.5 × 20 cm α-casein column at 2 ml/min. The column was washed with 2.5 column volumes of equilibration buffer. Bound proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in a total volume of 0.7 liters of Buffer B at 1.5 ml/min. The collected fractions (10 ml each) were analyzed by Western blotting.

Step 4: S Sepharose Fast Flow Chromatography—The immunoreactive fractions were dialyzed against Buffer C (conductivity < 1.5 millisiemens) and applied to a 2.5 × 6 cm S Sepharose Fast Flow column at 2–3 ml/min. The column was washed with 60 ml of Buffer C and then developed with a linear NaCl gradient (0–0.5 M) in 210 ml of equilibration buffer at 1 ml/min. The immunoreactive fractions (42–51; 30 ml total) were dialyzed for 3 h in Buffer C. The desalted sample was then concentrated using a 0.8 ml of S Sepharose Fast Flow mini-column. Elution was achieved in one step using 1 ml of Buffer C supplemented with 1 M NaCl.

Step 5: Gel Filtration on Superose 12 Preparative Grade—A 1.6 × 50 cm S12 column was preequilibrated in Buffer B supplemented with 50 mM NaCl. The sample from step 4 was applied to the S12 column and chromatographed at 0.3 ml/min. Fractions of 1 ml were collected, and 30-μl aliquots were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions showing the highest purity (typically 40–43) were pooled and stored at −70 °C.

Microsequencing

Purified NAP-185 (90 μg) was precipitated with 0.16 volumes of 50% trichloroacetic acid. The precipitate was washed twice with 1 ml of a 1:1 (v/v) mix of ether/ethanol to remove the detergent, and S-pyridyl ethylation was performed as described (19). After endoproteinase Lys-C cleavage (1:50 molar ratio), the sample was acidified and applied to a C8 microbore column (2.1 × 30 mm; RP-8, 5 μm, Pierce Chemical Co.) preequilibrated in 0.05% trifluoroacetic acid. Separation of the resulting peptides was performed on an ABI 140A/785A system using a linear gradient (0–100%) of 0.04% trifluoroacetic acid, 80% acetonitrile in 60 min. Selected fractions were analyzed for purity by electrospray mass spectrometry (Sciex API-III), and those containing a single peptide spe-

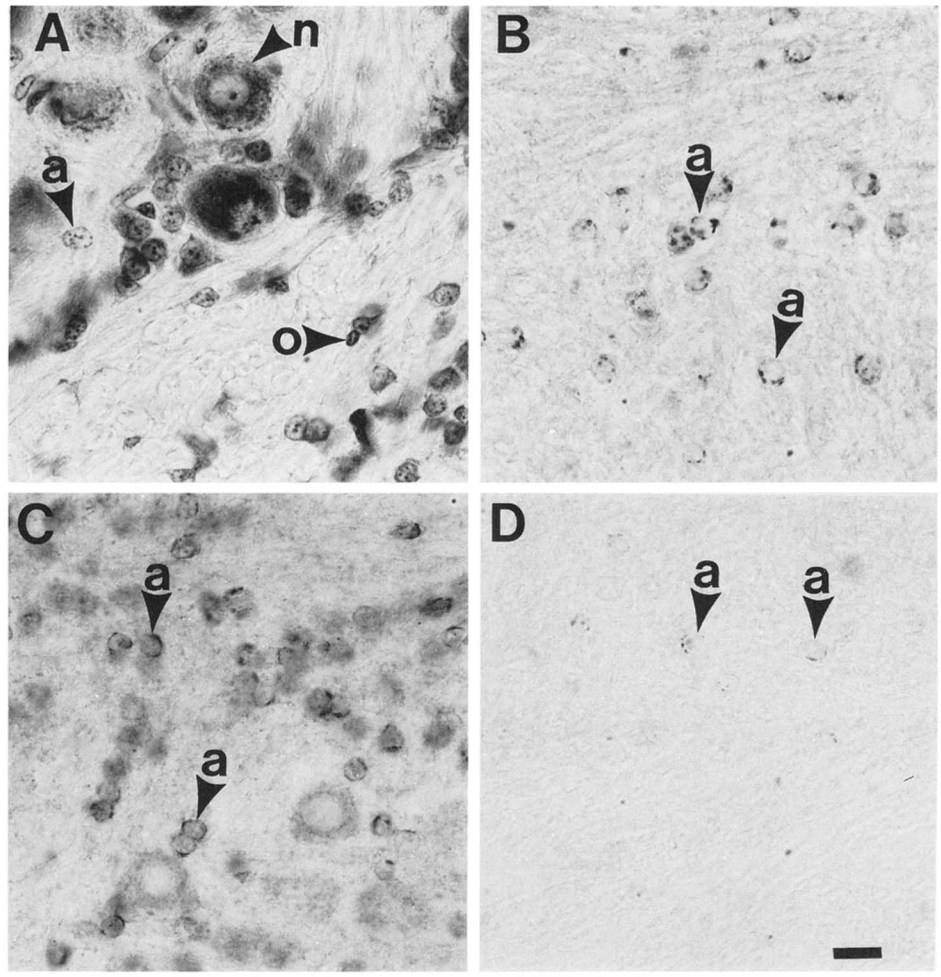

FIG. 1. **NAP-185 is localized in astrocytes in the chick brain stem.** *A* represents a section of the nucleus magnocellularis stained with thionin (29). The neurons (*n*) are surrounded by astrocytes (*a*) and oligodendrocytes (*o*). The latter are differentiated by their size and shape. Oligodendrocytes are smaller and more oval shaped. Alternate sections were stained either with 5501 Ab (*B*) or with 2844 Ab (*C*). Competition of the specific 5501 stainng was achieved by the addition of purified NAP-185 to the primary staining solution at a final concentration of 100 μg/ml (*D*). *Bar* = 10 μm.

cies were subjected to automated Edman degradation on a model 470A gas phase protein sequenator (Applied Biosystems Inc.) according to the manufacturer's protocol.

Other Methods Used

Protein concentration was determined according to Bradford (20). Protein tyrosine phosphatase activity was assayed using the peptide substrate ENDY(P)INASL as described previously (21). SDS-polyacrylamide gel electrophoresis analysis was performed with 10% polyacrylamide gels, according to Laemmli (17).

RESULTS

Identification of NAP-185 as an Astroglial Marker in Chick Brain Stems—Using an affinity purified antiserum (5501 Ab) directed against the catalytic domain of T-cell protein tyrosine phosphatase (22) to perform immunocytochemical analyses of chick brain stems, we identified a population of neuroglial cells strongly resembling astrocytes (Fig. 1*B*). A comparison between Fig. 1, *A* and *B* reveals that the 5501 Ab does not label all astrocytes but only a specific subpopulation. Unexpectedly, when brain stem immunoprecipitates were analyzed by Western blotting, we did not find the 48-kDa band as expected for T-cell protein tyrosine phosphatase (Fig. 2, *lane 1*), but rather we found a protein with a molecular mass of 185 kDa (Fig. 2, *lane 2*) that we named NAP-185. This 185-kDa protein was purified to near homogeneity and microsequenced, and an antibody (2844) recognizing a peptide derived thereof was generated. This antibody labeled a similar subpopulation of astrocytes (Fig. 1*C*). Furthermore, the 5501 staining could be competed by incubating the sections in the presence of purified NAP-185 protein (Fig. 1*D*). Taken together, these results show that this 185-kDa protein is responsible for the astrocytic pattern observed and that it is restrictively expressed in neuroglial cells in the chick brain stem.

Purification of NAP-185—An overview of the purification is presented in Fig. 3. NAP-185 was monitored during purification by Western blot analysis using the 5501 antiserum. The protein tyrosine phosphatase activity profile of each chromatographic step was assessed with the peptide ENDY(P)INASL. Approximately half of the total protein tyrosine phosphatase activity was adsorbed to DE52; as shown in Fig. 3*A*, several peaks of protein tyrosine phosphatase activity were resolved. Immunoblotting with 5501-antiserum across the entire profile showed that the main reactive species was the 185-kDa protein previously observed, and peak fractions were identified (60–95). At this stage of the purification, it was not possible to correlate the immunoreactive NAP-185 fractions with any of the protein tyrosine phosphatase activity peaks, indicating that NAP-185 was not one of the major protein tyrosine phosphatases present. Furthermore, the sequential loss of total protein tyrosine phosphatase activity throughout the purification suggests that despite its immunological relationship with T-cell protein tyrosine phosphatase, NAP-185 might not be a protein tyrosine phosphatase at all.

Indeed, the protein tyrosine phosphatase activity in fractions 40 to 43 of the Superose 12 column was almost undetectable, while NAP-185 was highly enriched (Figs. 3*D* and 4).

Microsequencing of NAP-185—In order to identify this protein and to compare its sequence with that of other proteins, internal sequencing was performed. To this end, fractions 40–43 from the Superose 12 sizing column were pooled (S12 pool) and chromatographed on Mono Q if necessary. Peptides

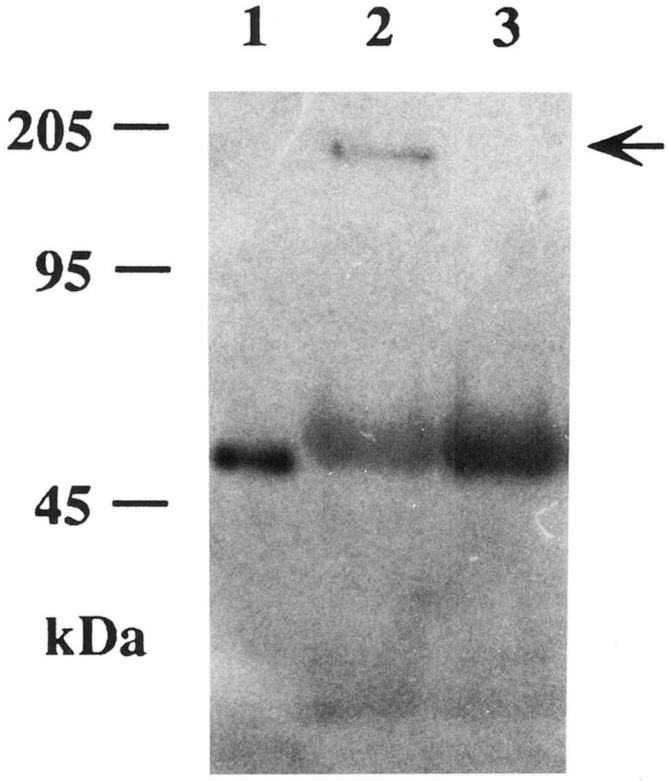

FIG. 2. **Western blot analysis of immunoprecipitates from chicken brain extracts.** Immunoprecipitates were generated either with 5501 antiserum (lane 2) or with preimmune serum (*lane 3*) as described under "Experimental Procedures." The proteins were separated on a 10% polyacryamide gel and transferred to nitrocellulose. After incubation with antibodies specific for the catalytic domain of T-cell protein tyrosine phosphatase (5501 antiserum), immunoreactive bands were visualized with iodinated protein A (0.1 μCi/ml). *Lane 1* contains 30 ng of purified T-cell protein tyrosine phosphatase standard.

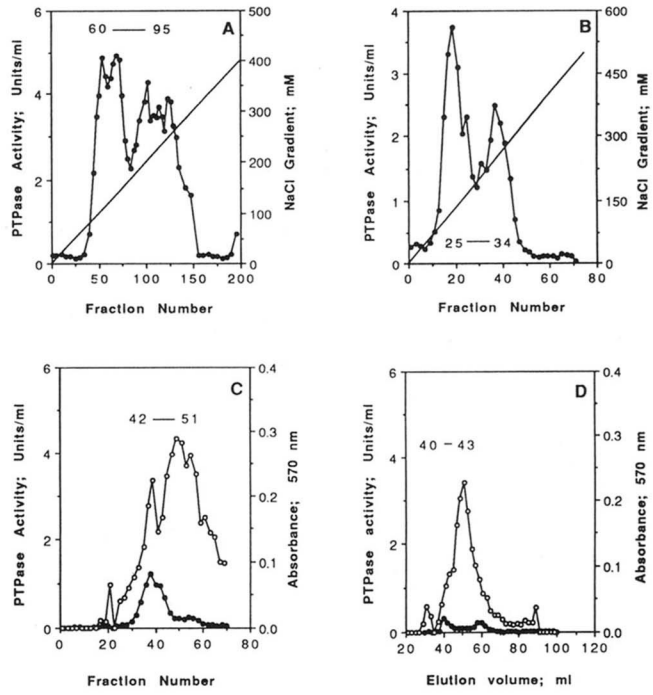

FIG. 3. **Chromatography of protein tyrosine phosphatase activity on consecutive columns.** *A*, diethylaminoethyl-cellulose; *B*, α-casein-Sepharose 4B; *C*, S Sepharose Fast Flow; *D*, Superose 12. The enzyme activity was assayed with ENDY(^{32}P)INASL substrate (●). The protein concentration profile was assessed by the method of Bradford (○). The *horizontal bars* in each graph indicate the fractions pooled; the *left number* denotes the first fraction of the pool. See "Experimental Procedures" for details.

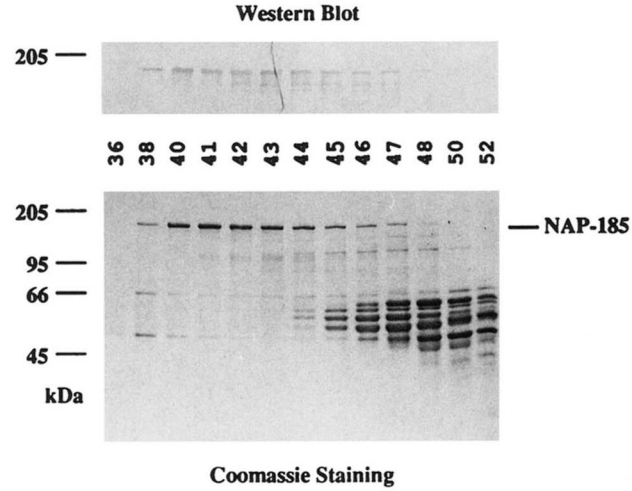

FIG. 4. **SDS-polyacrylamide gel electrophoresis and Western blot analysis of the fractions obtained in the Superose 12 purification step.** Aliquots (3 or 30 μl) of fractions 36–52 were subjected to 10% SDS-polyacrylamide gel electrophoresis. One gel (3 μl samples) was transferred to nitrocellulose for 5501 staining (*top panel*), while the other was used for Coomassie staining (*bottom panel*). No immunoreactive proteins were observed below 100 kDa.

derived from NAP-185 were then obtained as described under "Experimental Procedures." The amino acid sequences of these peptides are shown in Fig. 5. The data were obtained from fractions containing at least 80 pmol of peptide, thus eliminating the possibility that they were derived from contaminating proteins. A search of the SwissProt and GenEMBL data bases revealed that peptides D and E share sequence identity (96 and 78% respectively) with AP180 (23, 24), a clathrin-associated protein. Peptide B shows a 94% sequence identity with the microtubule associated protein *tau* (MAP-*tau*, Refs. 25 and 26). The other two peptides (A and C) revealed no significant relationship to any other sequence listed in the data bases. These data, together with the strong likelihood that the five peptides were derived from a single molecule, suggest that NAP-185 is a new protein sharing some sequences with AP180 and the MAP-*tau* proteins.

NAP-185 Is Related to AP180 and MAP-tau—In order to further corroborate that the peptides obtained above belong to a single protein, immunoprecipitation experiments were performed. Purified NAP-185 (S12-pool) was incubated with solid-phase coupled antibodies specific for AP180, and the resulting immunecomplexes were blotted with mAb AP180-I and affinity-purified 2844 or 5501 antibodies. A 185-kDa protein could be immunoprecipitated and detected with mAb AP180-I (Fig. 6, *lanes 2* and *5*). That same protein was detected with the 2844 antiserum showing that the B-peptide sequence is present in the protein (Fig. 6, *lanes 1* and *4*). Immunoprecipitates generated with the 5501 antiserum also displayed a 185 kDa band when probed with mAb Ap180-I (data not shown).

Tissue Distribution of NAP-185—Western analysis of different chick tissue extracts revealed that NAP-185 is not restricted to the brain (Fig. 7). In fact, its highest level of expression was found in kidney and stomach while gizzard, liver, and brain expressed lower amounts, and heart and skeletal muscle essentially none. The high molecular weight band observed in these blots was confirmed to be NAP-185 since the 2844 antiserum could also detected it (data not shown). The preimmune serum did not immunoprecipitate this protein as shown in Fig. 7 (*lane 1*).

NAP-185 Is Expressed in Nonproliferating Neuroglia—In the neonatal chick brain stem, there is a low basal rate of astrocyte

A

Name	Sequence	Peptide (pmoles)	Molecular Weight Calculated	Observed
Peak A	VQILNK	100	714.45	713.40
Peak B	VQIVYKPVDLSHVTSK	120	1813.02	1812.40
Peak C	DGGEDRDAAVEEAVLGTGGCRTPK	120	2508.17	2508.40
Peak D	GLGSDLDSSLANLVGNLGISGTTSK	305	2376.23	2375.98
Peak E	VDSTGVIDLFGDAFGSQA	80	-	-

B

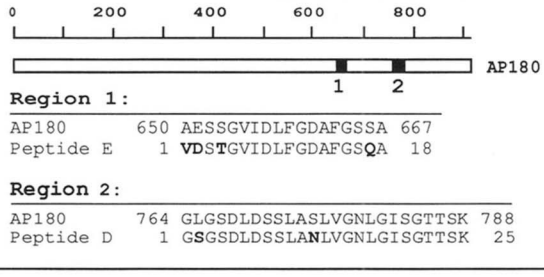

FIG. 5. **Partial amino acid sequences from NAP-185.** The primary amino acid sequence was obtained from various peptides as described under "Experimental Procedures" and is presented in *panel A*. *Peaks A–D* were analyzed by both gas phase sequencing and electrospray mass spectrometry. The sequence of peptide A was inferred from a mixture of peptides using a combination of tandem mass spectrometry and gas phase sequence analysis. To calculate the molecular weights of the peptides, a value of 227.08 was used for cysteine residues since the protein was modified by *S*-pyridylethylation. There was insufficient material to sequence to the end of peptide E. The amount in the peptide peaks was determined from sequencing data. *Panel B* shows the sequence alignment and the relative position of peptides D and E on the AP180 molecule (23). The amino acid residue numbers correspond to the sequence deposited in the EMBL Sequence Data Library under the accession number X68878.

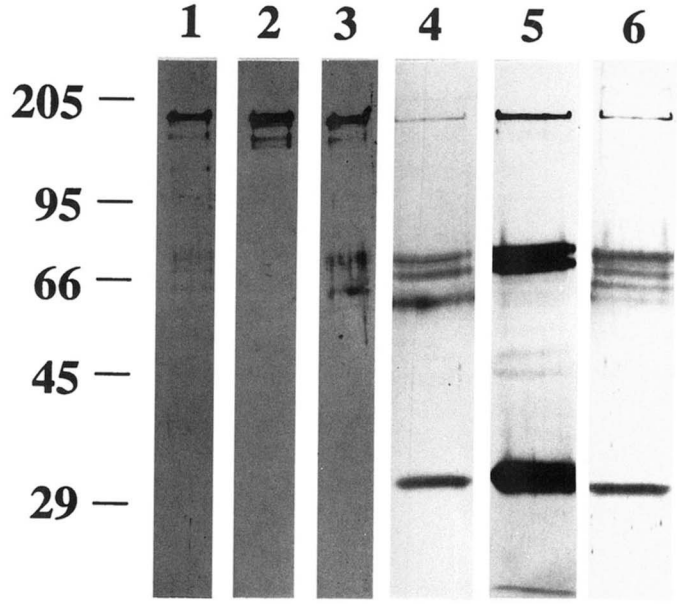

FIG. 6. **Western blot analysis of AP180-I immunecomplexes of purified NAP-185 (S12 pool).** *Lanes 1–3* represent aliquots of 500 ng of NAP-185. Excess protein was loaded to detect any contaminating immunoreactive proteins. *Lanes 4–6* represent immunoprecipitates generated from 500 ng of NAP-185 with the mAb AP180-I. The antiserum 2844 was used in the immunodetection step in *lanes 1* and *4*. *Lanes 2* and *5* were probed with the mAb AP180-I. The 5501 antiserum was used for *lanes 3* and *6*. Immunoreactive proteins were visualized using goat anti-rabbit or goat anti-mouse alkaline phosphatase-coupled antibodies. The protein bands around the 66 and 29 kDa markers, observed in *lanes 4–6*, correspond to the heavy and light chain of the precipitating antibodies.

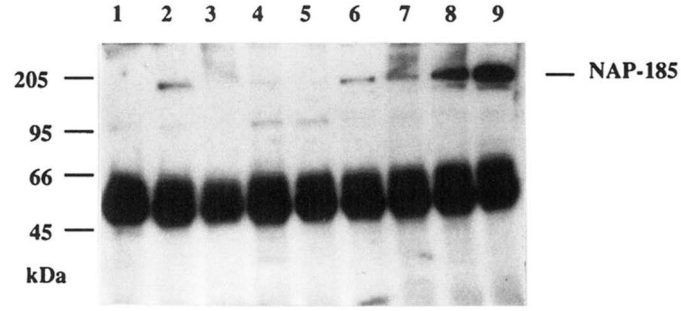

FIG. 7. **Tissue distribution of NAP-185.** Tissue extracts were prepared and immunoprecipitated with 5501 antiserum as described under "Experimental Procedures" with the exception of *lane 1* representing brain tissue immunoprecipitated with preimmune rabbit antiserum. The 5501 antiserum was used to probe the nitrocellulose blot. Immunoreactive proteins were detected with iodinated protein A (0.1 μCi/ml). *Lane 2*, brain; *lane 3*, intestine; *lane 4*, skeletal muscle; *lane 5*, heart; *lane 6*, liver; *lane 7*, gizzard; *lane 8*, stomach; *lane 9*, kidney. The 50 kDa band corresponds to the heavy chain of the immunoprecipitating antibodies.

proliferation (15). Deafferentation of the nucleus magnocellularis achieved by cochlea removal results in an hyperplastic and hypertrophic response of astrocytes which become "reactive" in that area. In order to study the fate of NAP-185 during reactive astrocytosis, glial cells in the nucleus magnocellularis were examined 2 days after cochlea removal when glial proliferation is increased. Double-labeling immunocytochemistry was performed with anti-BrdU (a thymidine analog that incorporates into the DNA of replicating cells) and 5501 antibodies. Expression of NAP-185 was observed restrictively in nonproliferating cells, as shown in Fig. 8. The dark nuclei revealed by anti-bromodeoxyuridine antibodies represent actively proliferating cells (*arrowhead*) within the chick brain stem. When the same sections were probed with 5501 Ab, staining was always strictly restricted to nondividing cells (*arrows*).

DISCUSSION

The identification of new biochemical markers for neuroglial cells in the central nervous system would be most valuable to our understanding of glial functions. This report suggests that NAP-185 may serve as such a marker. While not restrictively expressed in brain (Fig. 7), NAP-185 appears to be specifically associated with a distinct subpopulation of nonneuronal cells in the chick brain stem (Fig. 1, *B* and *C*).

The specific neuroglia subtype expressing NAP-185 has been difficult to identify unequivocally due to the lack of chick-specific astrocyte markers. All of the antibodies tested and directed against common glial proteins such as OX42, glial fibrillary acidic protein, glutamine synthetase, S-100, carbonic anhydrase C, and vimentin failed to identify the subpopulation of glial cells labeled with the 5501 antiserum. Therefore, immunocytochemical confirmation that this protein is specifically found in a particular glial subtype could not be obtained by double-labeling experiments. Nonetheless, it is reasonable to assume that it is expressed in astroglial cells based on ultrastructural observations. Indeed, thionin staining of chick brain stem tissue shows that NAP-185 is present in cells containing large, round, and pale nuclei that are characteristic for astrocytes (Fig. 1*A*, *a*). Oligodendrocytes, which display smaller and oval shaped nuclei (Fig. 1*A*, *o*) are therefore unlikely candidates. The even distribution, observed at lower magnification and the relatively high density of cells, identified by antibodies 5501 and 2844 further suggests its astrocytic nature. Finally, it is very unlikely that NAP-185 is produced in microglial cells since staining for the microglial marker OX42 (27) revealed

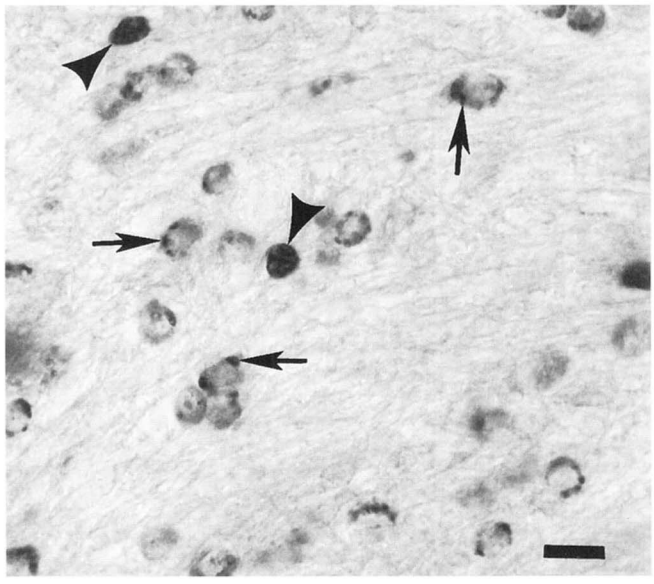

FIG. 8. **In situ localization of NAP-185 visualized by double-labeling immunocytochemistry.** This figure shows that NAP-185 is expressed in nonproliferating cells. NAP-185 was detected with 5501 Ab (*small arrowhead*). Dividing astrocytes were labeled with anti BrdU antibodies (*large arrow*). For staining details see "Experimental Procedures." *Bar* = 10 μm.

only a few labeled cells in sections of the chick brain stem and since this labeling did not correlate with 2844 or 5501 staining (15). Therefore, the data strongly suggests that NAP-185 is indeed expressed in a specific subtype of astrocytes and might serve as a marker for those cells.

The specific cellular localization of NAP-185 within the brain as well as its immunological relationship to T-cell protein tyrosine phosphatase triggered our efforts in purifying this particular protein. Immunoglobulins purified on a T-cell protein tyrosine phosphatase affinity column recognized both NAP-185 and T-cell protein tyrosine phosphatase, indicating that they must share one or more related epitopes. However, use of the 5501-antiserum to monitor purification as well as determination of the protein tyrosine phosphatase activity profile of each chromatographic step indicated that purified NAP-185 had no protein tyrosine phosphatase activity. On the other hand, the absence of a required positive regulator, such as a ligand, or the loss of a regulatory subunit of NAP-185 cannot be entirely ruled out. The extent of purification was roughly estimated to be around 10,000-fold based on total protein and assuming a final recovery of 10%.

The microsequence information was obtained from an initial batch of 500 pmol of NAP-185, and each of the peptide fractions sequenced contained at least 80 pmol. Consequently, the sequences analyzed were almost surely derived from the same molecule. Nevertheless, in view of the similarity between the molecular weights of NAP-185 and AP-180, as well as the sequence overlap, it became necessary to demonstrate that the two proteins were different. As shown in Fig. 6 the immunoprecipitated 185-kDa protein could be detected with all three antisera tested. More specifically, the 185 kDa band reacted with the affinity purified 2844 antibodies. This antiserum was generated against peptide B, specifically selected because its sequence is not present in AP180. We can also rule out that peptide B results from a contaminating MAP-*tau* protein since no prominent band was detected by Western analysis in the 35–55 kDA range corresponding to *tau* (Fig. 6, *lane 1*). Tissue distribution of NAP-185 also indicates that they are different proteins, while AP180 has been shown to be exclusively restricted to synapses (28), NAP-185 is also expressed in kidney and stomach. Furthermore, staining of chick brain stems with mAb AP180-I specifically identified the neurons in the nucleus magnocellularis and did not label astrocytes (data not shown). Taken together, our results show that NAP-185 is a novel protein possibly related to the AP180 family.

As of now, the exact function of AP180 has not been elucidated. However, its specific synaptic localization and its ability to promote clathrin aggregation *in vitro* suggest that this protein serves as an adaptor molecule between clathrin and membrane receptors and participates in the endocytotic recycling of synaptic vesicles by a coated pit mechanism. It is possible that NAP-185 plays a similar role in nonneuronal cells. The recent discovery that the proteins involved in the fusion of synaptic vesicles with the plasma membrane exist in multiple isoforms, some expressed only in neuronal and neuroendocrine cells and others in other tissues, supports the idea that these proteins play a more general role in membrane fusion events (30). Moreover, synaptic-like microvesicles have been identified in a number of nonneuronal cells (31, 32, 33) as the storage site of the classical nonpeptidergic neurotransmitters. While the presence of synaptic-like microvesicles in astrocytes has not yet clearly been established, these cells are known to release endogenous amino acid neuromodulators such as glutamate, aspartate, glycine, taurine, and γ-aminobutyric acid (34). Very recently, glutamate of astrocytic origin has been implicated in astrocyte-neuron signaling (35). Parpura and co-workers (35) could indeed demonstrate that bradykinin-induced release of glutamate from astrocytes causes an internal elevation of calcium in co-cultured neurons. Since solitary neurons did not respond to the bradykinin treatment and glutamate receptor antagonists could block the increase in neuronal intracellular calcium, the data suggest that astrocytes can establish synaptic-like contacts with neurons. If so, it will be important to determine the specific function of NAP-185 in glial cells.

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