Neuron-specific Membrane Glycoproteins Promoting Neurite Fasciculation in *Aplysia californica*

Flavio Keller*‡ and Samuel Schacher‡

*Howard Hughes Medical Institute and ‡Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, New York 10032

Abstract. We have generated a library of mouse monoclonal antibodies against membrane proteins of the nervous system of the marine snail *Aplysia californica*. Two of these antibodies, 4E8 and 3D9, recognize a group of membrane glycoproteins with molecular masses of 100–150 kD. We have called these proteins apl00, from the molecular mass of the most abundant species. Based on Western blots, these proteins appear to be specific for the nervous system. They are enriched in the neuropil of central nervous system ganglia, and are present on the surface of neurites and growth cones of neurons in culture. They are not expressed on the surface of nonneuronal cells. Staining of living cells with fluorescently labeled mAb demonstrates that the epitope(s) are on the outside of the cell. The antibodies against the proteins defasciculate growing axons and alter the morphology of growth cones, but affect much less adhesion between neuritic shafts. In addition, the level of expression of these molecules appears to correlate with the degree of fasciculation of neurites. These observations suggest that the apl00 proteins are cell adhesion molecules that play a role in axon growth in the nervous system of *Aplysia*. The fact that they are enriched in the neuropil and possibly in varicosities suggest that they may also be relevant for the structure of mature synapses.

THERE is a large body of evidence that glycoproteins expressed on the surface of growing axons are important for axon guidance during development (Fischer et al., 1986; Bixby et al., 1987; Rathjen et al., 1987; Harrelson and Goodman, 1988; Matsuraga et al., 1988; Tomaselli et al., 1988; Keller et al., 1989; Ruegg et al., 1989; Snow et al., 1989; Furley et al., 1990). Although the organization of vertebrate and invertebrate nervous systems are different, the molecular strategies involved in the establishment of the final pattern of neuronal connections appear to be similar (Jessell, 1988).

The nervous system of *Aplysia* is potentially interesting for the investigation of molecular processes associated with the development of the nervous system, and of the role of adhesion molecules in particular, because of the possibility of maintaining identified neurons in culture, where they regenerate neurites and reestablish appropriate chemical synapses (Camardo et al., 1983; Schacher and Proshansky, 1983; Rayport and Schacher, 1986).

Aplysia neurons in culture are a useful model for a detailed study of growth cone motility (Goldberg and Burmeister, 1989), and would therefore be a suitable system to study the role of cell adhesion molecules in axon navigation. Furthermore, due to their large size *Aplysia* neurons are amenable to repeated injection of fluorescent tracers, allowing a detailed study of axon growth and synapse formation (Glanzman et al., 1989) and long-term synaptic modulation (Glanzman et al., 1990). The large size of the neurons might also allow the transient alteration of the level of gene products in single neurons by injection of nucleic acids (Dash et al., 1990). We therefore set out to generate monoclonal antibodies against membrane proteins expressed in the nervous system of juvenile animals, with the aim of identifying proteins expressed on the surface of neurons, enriched in the neuropil of central nervous system (CNS)[1] ganglia, and present on neurites and growth cones of neurons in culture.

We isolated two mAbs (4E8 and 3D9) that recognize the same group of membrane proteins. We used these antibodies for a biochemical and immunocytochemical characterization of the proteins, and for perturbation studies of axon outgrowth and fasciculation in cultures of identified *Aplysia* neurons. The results are consistent with the idea that these proteins are involved in axon fasciculation. In addition, their presence in the adult CNS and enrichment in the neuropil suggest that they might have a functional role at mature synapses.

Materials and Methods

Culture of Aplysia Neurons

The cell culture techniques and media used to isolate and maintain iden-

1. *Abbreviations used in this paper*; CNS, central nervous system; LUQ, left upper quadrant; RUQ, right upper quadrant.

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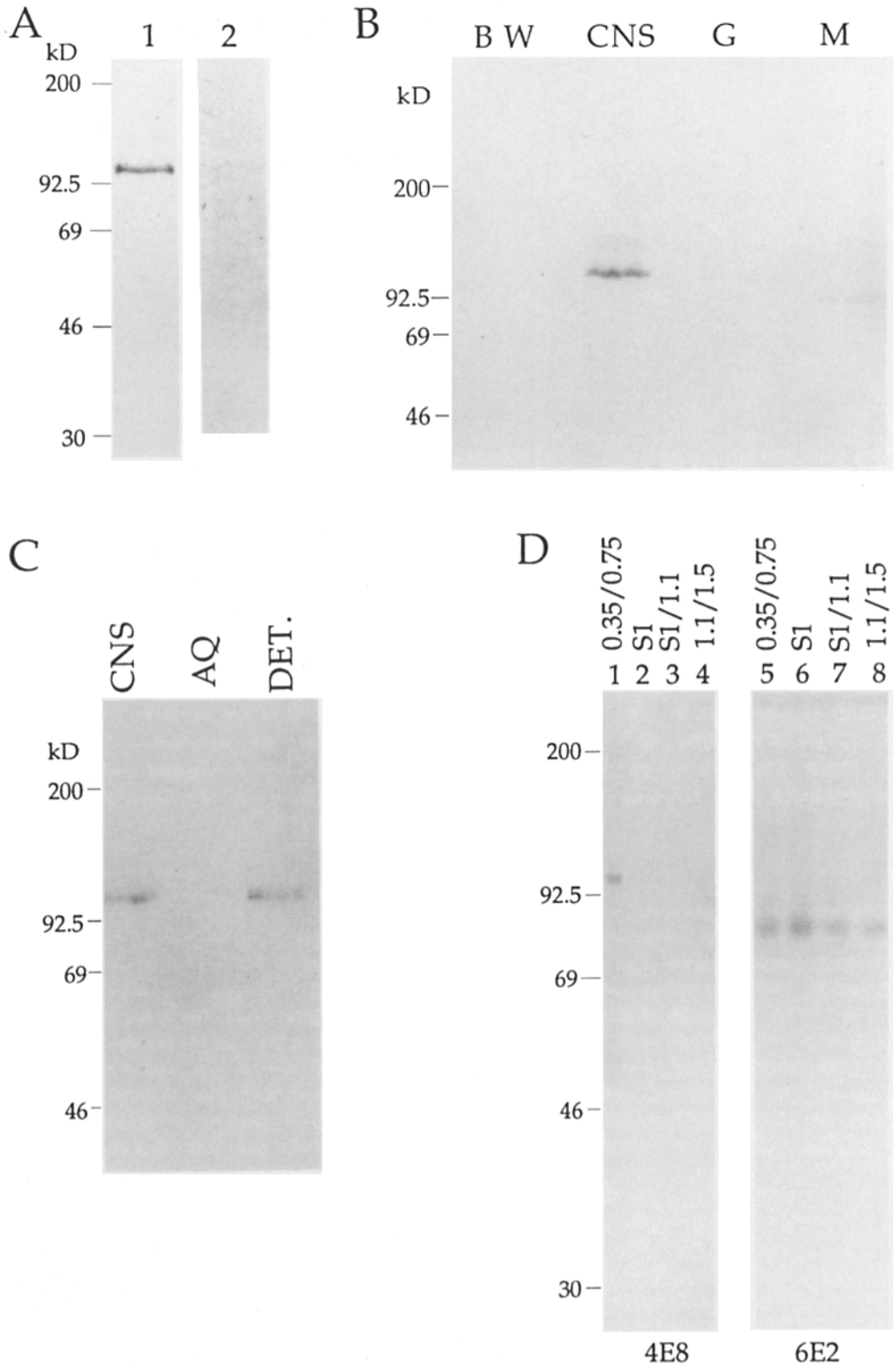

Figure 1. (*A*) Western blot of *Aplysia* CNS stained with mAb 4E8 (lane *1*) or nonimmune mouse IgG (lane *2*) at comparable dilutions (1:25). The same amount of total protein (21 μg) was present per lane. mAb 4E8 specifically stains a band at 100 kD. The position of molecular weight markers is shown on the left (myosin, 200 kD; phosphorylase b, 92.5 kD; bovine serum albumin, 69 kD; ovalbumin, 46 kD; carbonic anhydrase, 30 kD). (*B*) Western blot of different tissues from *Aplysia*, stained with mAb 4E8. Equal amounts of protein (40 μg) were loaded in each lane. *BW*, body wall; *CNS*, CNS ganglia; *G*, gut wall; *M*, musculature of the buccal mass. Note the prominent 100-kD band in the CNS, which is absent in the other tissues. A faint band at ~80 kD appears in the buccal muscle. (*C*) Detergent phase partitioning of the 100-kD antigen. A membrane preparation from CNS ganglia was extracted with Triton X-114, separated by SDS-PAGE, blotted, and stained with mAb 4E8. *CNS*, unextracted membranes (29 μg); *AQ*, aqueous phase (14 μg); *DET*, detergent phase (6 μg). (*D*) Western blot of sucrose gradient fractions of *Aplysia* CNS stained with mAb 4E8 or mAb 6E2. In lanes stained with mAb 4E8, 1.3 μg protein was loaded in each lane. 26 μg protein was loaded in the lanes stained with mAb 6E2.

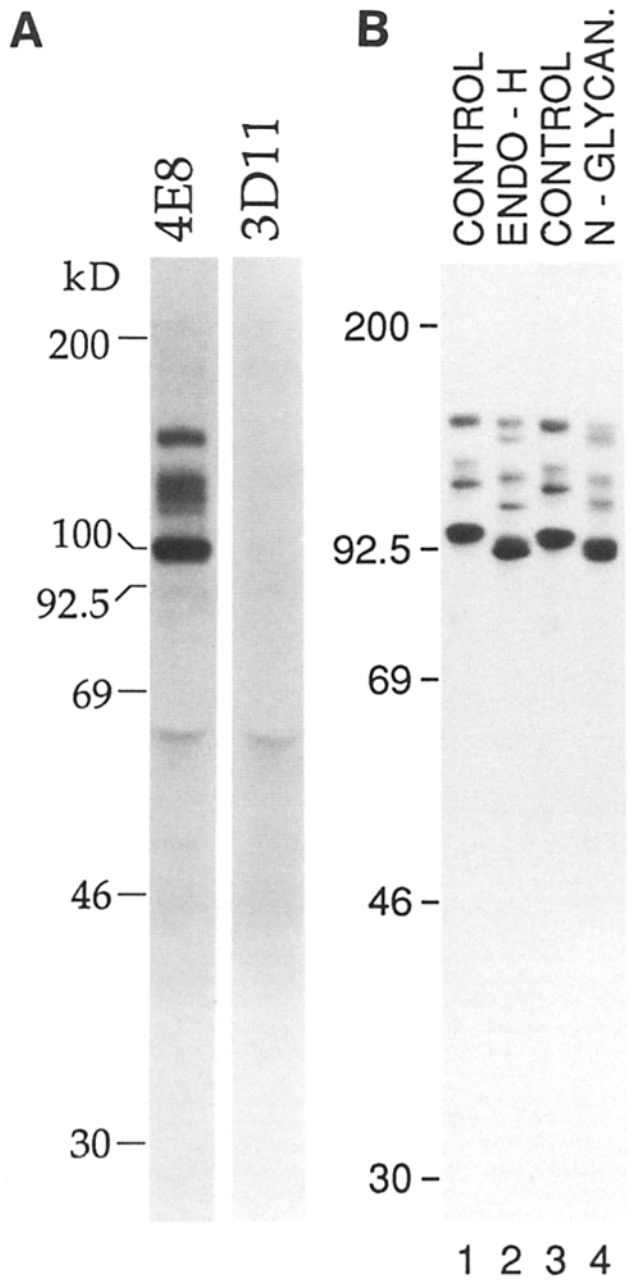

Figure 2. (*A*) Immunoprecipitation of [^{35}S]methionine-labeled CNS ganglia with mAb 4E8 or mAb 3D11. mAb 4E8 precipitates two sharp bands at 100 and 150 kD. In addition, there are several bands (probably three) between 110 and 130 kD. Note the absence of any bands between 100 and 200 kD after immunoprecipitation with the control mAb (3D11). (*B*) Treatment of proteins immunoprecipitated by mAb 4E8 with two different *N*-glycosidases: endoglycosidase H (lane *2*) and *N*-glycosidase F (*N*-glycanase, lane *4*). The respective controls (digestion buffer without enzyme) are shown in lanes *1* and *3*.

tified neurons of *Aplysia californica* have been described (Schacher and Proshansky, 1983; Schacher, 1985; Flaster et al., 1986; Rayport and Schacher, 1986). The following cells were isolated from juvenile animals: left upper quadrant (LUQ) cells (L2-L6), right upper quadrant (RUQ) cells (R3-R13), motor neuron L7, L11, and cells from the RB cluster. The mechanosensory cells from the LE cluster and the neuroendocrine bag cells were isolated from adult ganglia. The cells were plated in dishes coated with poly-*l*-lysine (Sigma Chemical Co., St. Louis, MO) containing culture medium with or without various mAbs.

Immunization, Fusion, and Screening

Balb/c mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized by footpad immunization with a membrane preparation from abdominal ganglia and pleural-pedal ganglia of juvenile (1–2 g) *Aplysia* (Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, Miami, FL). Membranes were prepared by homogenizing the ganglia from 10 animals in 0.1 M sodium phosphate buffer, pH 7.4, containing 5 mM EDTA and 50 μg/ml PMSF as protease inhibitors. The homogenate was centrifuged at 1,000 *g* for 10 min and membranes were pelleted from the supernatant by centrifugation at 200,000 *g* for 60 min. The membrane pellet was resuspended in phosphate buffer and emulsified with an equal volume of complete Freund adjuvant (Gibco Laboratories, Grand Island, NY). The emulsion was injected into one footpad. Each mouse received ~10 μg membrane protein per injection on days 1, 5, 9, and 13 (Hockfield, 1987). Footpad immunization was required because of the small amount of protein.

On day 14 popliteal lymph node lymphocytes were fused with NS-1 myeloma cells by addition of polyethylene glycol 1500 (Boehringer Mannheim Biochemicals, Indianapolis, IN). Hybridomas were selected in Dulbecco's modified Eagle's medium (Gibco Laboratories), supplemented with 20% fetal calf serum (Hyclone Laboratories, Logan, UT) and HAT selection medium (Gibco Laboratories).

The supernatants were screened on sections from abdominal ganglia and pleural-pedal ganglia of adult (100–200 g) *Aplysia*. Sections were prepared as described by Swanson et al. (1986). The sections were rinsed with PBS and incubated sequentially with PBS containing 0.1 M glycine (10 min), PBS with 10% normal goat serum (Gibco Laboratories) for 30 min, and then with hybridoma supernatant overnight at 4°C. Bound antibodies were visualized by incubation with Fab fragments of rabbit anti-mouse immunoglobulins (Cappel Laboratories, Malvern, PA) diluted 1:500 in PBS/goat serum, followed by TRITC-labeled Fab fragments of goat anti-rabbit Fab immunoglobulins (Cappel Laboratories) 1:50 in PBS/goat serum. Sections were mounted in 10% PBS/90% glycerol and viewed with a Leitz ortholux fluorescence microscope.

Many hybridomas showed a general staining of the sheath of the ganglia. These antibodies were discarded. The hybridomas whose supernatants selectively stained the surface of nerve cells and the neuropil were expanded and tested on cultured *Aplysia* neurons. Neuronal cultures (mixed cell type, see above) were fixed for 2 h with 4% paraformaldehyde/30% sucrose (see above) and stained as for tissue sections.

Hybridomas staining the neuropil of ganglia as well as neurites of cultured nerve cells were subcloned by the method of limiting dilution. The antibody subtype was determined by the mouse clonotyping system (Amersham Corp., Arlington Heights, IL).

Purification, Fragmentation, and Fluorescent Labeling of mAbs

mAbs 4E8 (IgG2a), 3D9 (IgG1), and 3D11 (IgG3) were purified from ascites fluid on immobilized protein A (Pierce Chemical Co., Rockford, IL). Mice were preinjected intraperitoneally with Pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Corp., Milwaukee, WI). mAb 6E2 (IgM) was purified from ammonium sulfate precipitated culture supernatant by gel filtration on a Superose 6 column (Pharmacia Fine Chemicals, Piscataway, NJ).

Fab fragments of IgG were prepared by digestion of the purified IgG with immobilized papain (Pierce Chemical Co.). Fc fragments were removed with immobilized protein A. The completeness of the digestion was checked with SDS-PAGE.

mAbs 4E8 and 3D9 were coupled with FITC (Sigma Chemical Co.) as described by Goding (1986). The molar fluorochrome/protein ratio was 4.1 for mAb 4E8 and 3.8 for mAb 3D9.

Immunocytochemistry

Sections were rinsed with PBS, incubated with PBS-glycine as indicated above, and then incubated with full-strength goat serum for 1 h. Purified mAb or nonimmune mouse IgG (control) were diluted with full-strength goat serum and sections were incubated overnight at 4°C. Bound antibody was revealed with rabbit anti-mouse antiserum (1:500), followed by peroxidase-labeled goat anti-rabbit antiserum (1:300). The use of full-strength serum appreciably reduced the background. The sections were then reacted

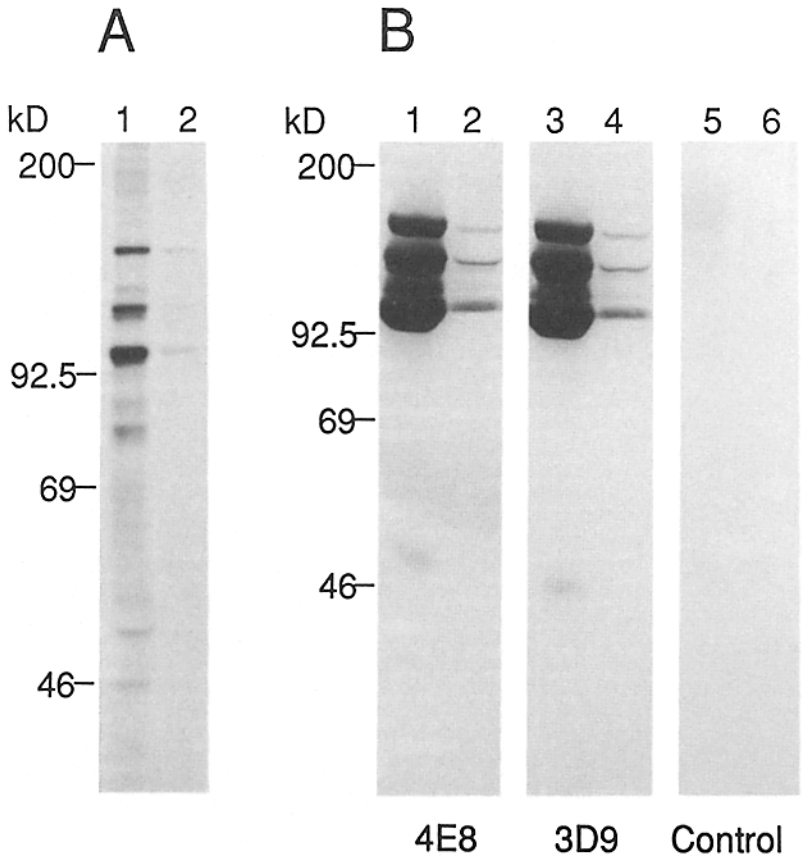

Figure 3. mAbs 4E8 and 3D9 recognize the same molecular species by immunodepletion and immunoaffinity isolation. (*A*) Immunodepletion experiment: CNS ganglia were labeled with [^{35}S]methionine and immunoprecipitated with mAb 3D9 with (lane *2*) or without (lane *1*) preabsorption with mAb 4E8. Preabsorption with mAb 4E8 strongly decreases the amount of 3D9-immunoprecipitable material. (*B*) Immunoaffinity isolation: ap100 was isolated from total CNS with a 4E8 affinity column (see Materials and Methods). Specifically bound proteins were eluted in 5-ml fractions at pH 2.6. The first fraction contained most of the protein. The second fraction contained a much smaller amount. After concentration, the first fraction was applied to lanes *1*, *3*, and *5* and the second to lanes *2*, *4*, and *6* of a 7.5% SDS-PAGE gel. Proteins were electroblotted and stained with mAb 4E8 (lanes *1* and *2*), mAb 3D9 (lanes *3* and *4*), or without primary antibody (lanes *5* and *6*). Bound antibody was revealed with alkaline phosphatase–conjugated goat anti-mouse antiserum. mAbs 4E8 and 3D9 recognize exactly the same immunoaffinity purified proteins.

with 0.01% diaminobenzidine, 0.6% nickel-ammonium sulfate, and 0.003% H_2O_2 in 50 mM Tris buffer, pH 7.4.

Photography

Specimens stained for immunofluorescence were photographed on Kodak P3200 film in a Leitz ortholux microscope. Specimens stained with immunoperoxidase were photographed on Kodak Tmax 100 film.

Immunocytochemistry of Live Cells in Culture

Cultures were rinsed first with perfusion medium consisting of a 1:1 mixture of L15 and artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH). Cultures were incubated for 30 min at 18°C with FITC-labeled 3D9 or 4E8 at 1:75 dilution (20 μg/ml). Cultures were rinsed with perfusion medium and examined on a Nikon Diaphot microscope equipped with a Dage 66 SIT and Dage 67 Newvicon cameras (Dage-MTI Inc., Wabash, MI). Fluorescent, Nomarski (DIC), and phase contrast images were stored on videotape using a Panasonic VCR and photographs made with a Mitsubishi video printer. For the double-label staining of sensorimotor cultures, the presence of a chemical synapse was first determined with standard intracellular recording techniques (Montarolo et al., 1986). The sensory cell was then injected with lissamine rhodamine (6% in distilled water; Molecular Probes Inc., Eugene, OR). The cells were then stained with the FITC-labeled mAbs. The two fluorescent images (rhodamine and fluorescein) of the same cellular regions were stored and photographed as described above.

Immunoprecipitation, SDS-PAGE, and Immunoblotting

For immunoprecipitation *Aplysia* CNS was labeled overnight at room temperature with [^{35}S]methionine (1,037 Ci/mmol, 0.5 mCi/ml; Amersham Corp.) in low methionine culture medium. The CNS was then homogenized in an ice-cold solution of 0.25 M sucrose and 25 mM Tris, pH 7.4, containing the following protease inhibitors: 3 mM EDTA, 1 mM EGTA, 5 mM benzamidine, 5 mM iodoacetamide, 0.1 mM PMSF, and 0.05 mM pepstatin A. After centrifugation of the homogenate at 1,600 *g* for 10 min to remove cell nuclei and sheath debris, the supernatant was centrifuged at 100,000 *g* for 1 h. The pelleted membranes were solubilized in 2.5% Triton X-100, 150 mM NaCl, and 25 mM Tris, pH 8.0, with the protease inhibitors listed above. 10 mg/ml BSA was included to reduce nonspecific binding. The solubilized membranes were spun at 100,000 *g* for 30 min to remove detergent-insoluble material and the supernatant was incubated overnight at 4°C with purified mAb. Antigen-antibody complexes were precipitated by addition of protein A-agarose beads (Pierce Chemical Co.). The pellet was washed first with 75 mM NaCl, 25 mM Tris, 1% Triton, and 50 mM Na_2HPO_4 (pH 8.0), and then with 1 M NaCl, 25 mM Tris, and 1% Triton (pH 7.4). Bound immune complexes were eluted by boiling the beads for 5 min in electrophoresis sample buffer and applied to 7.5% polyacrylamide gels (Laemmli, 1970). After electrophoresis, the gels were treated with Amplify (Amersham Corp.), dried, and exposed on Kodak X-Omat AR film at −70°C.

For Western blotting, CNS membranes were solubilized by boiling in 1% SDS. Total proteins were measured with the micro-BCA reagent (Pierce Chemical Co.). Proteins were electroblotted on Immobilon P (Millipore, Bedford, MA) and stained with the mAb followed by an alkaline phosphatase–conjugated goat anti-mouse antiserum (Promega Biotec, Madison, WI) as recommended by the manufacturer.

Analysis of Glycosylation

Whole CNS was labeled with [^{35}S]methionine and immunoprecipitated with mAb 4E8 as indicated above. The precipitated proteins were treated with *N*-glycanase (Genzyme Corp., Boston, MA) according to the instruction provided, Endo-H (0.8 U/ml in sodium cacodylate buffer, pH 6.0, 0.75% Nonidet-P40; Sigma Chemical Co.), or neuraminidase (from nonpathogenic *Streptococcus*, 1 U/ml in 100 mM sodium acetate buffer, pH 5.5, containing 0.05% BSA and 0.01 mM $CaCl_2$ [Genzyme Corp.]) for 6 h at 37°C. Control samples were incubated in buffer alone. Thereafter, the samples were processed for SDS-PAGE as described above.

As an alternate approach, *Aplysia* CNS was labeled with [^{35}S]methionine in the presence of 15 μg/ml tunicamycin (Sigma Chemical Co.) and analyzed by immunoprecipitation.

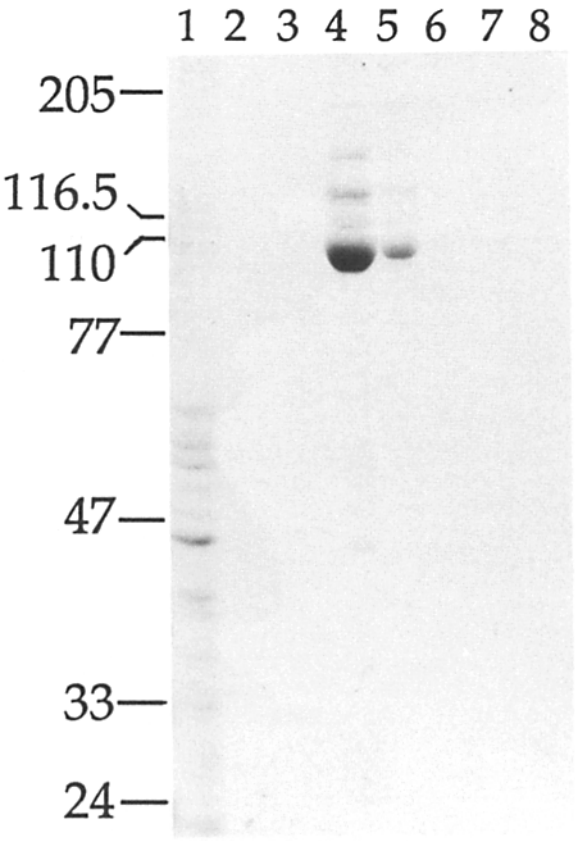

Figure 4. Immunoaffinity isolation of 4E8 antigen. A membrane extract of *Aplysia* CNS was run on an immunoaffinity column prepared by crosslinking mAb 4E8 to protein A immobilized on agarose. Specifically bound proteins were eluted as described in Materials and Methods. Eluted fractions were concentrated and applied to a 7.5% SDS-PAGE gel. The gel was stained with Coomassie blue.

Immunoaffinity Isolation

Affinity columns were prepared by crosslinking mAb 4E8 and 3D9 to protein A immobilized on agarose according to Schneider et al. (1982). *Aplysia* CNS membranes were prepared and solubilized as described above. The extract was passed over the columns overnight at 4°C. The columns were then washed with 10 vol of the following buffers: 0.5 M NaCl, 50 mM Tris, 1 mM EDTA, and 0.5% Triton, pH 8.0; 150 mM NaCl, 50 mM Tris, 1% Triton, and 0.1% SDS, pH 8.0; and 150 mM NaCl and 1% octyl-β-glucoside. Bound proteins were eluted either with 100 mM glycine buffer, pH 2.6, or with 50 mM diethylamine buffer, pH 11.6, containing 150 mM NaCl, 1% octyl-β-glucoside, and EDTA, EGTA, and benzamidine as protease inhibitors. 5-ml fractions were collected and neutralized immediately with 0.5 ml 1 M Tris, pH 9.5, or 1 ml 0.5 M NaH_2PO_4. Eluted fractions were further purified with SDS-PAGE. Elution at pH 2.6 yielded a preparation that appeared >90% pure. Elution at pH 11.6 yielded a less homogeneous preparation (several contaminating bands at lower molecular weight).

Results

Biochemical Analysis of Proteins Recognized by mAbs 4E8 and 3D9

The initial biochemical analysis was performed with mAb 4E8 (the first one isolated). Later, we isolated mAb 3D9, which crossreacts with the molecular species bound by mAb 4E8 (see below). Clone 3D9 tended to produce higher amounts of antibodies. A prominent band at 100 kD was stained by mAb 4E8 in Western blots of *Aplysia* CNS ganglia (Fig. 1 *A*, lane *1*). The staining is specific, since nonimmune mouse IgG did not show any staining (Fig. 1 *A*, lane *2*). This band appears to be specific for the nervous system (Fig. 1 *B*, *CNS*), since all nonneural tissues tested so far (body wall, gut, buccal muscle, and sperm) do not show any corresponding bands (Fig. 1 *B*). The connectives between the pleural and abdominal ganglia and other peripheral nerves also express the 100-kD band (data not shown). Depending on the amount of protein loaded onto the gels and the antibody concentration, mAb 4E8 (and 3D9) also stained bands at molecular masses higher than 100 kD (110, 120, and 150 kD). These bands can be seen as faintly stained in Fig. 1 *B* (*CNS*), but are much more prominent after immunoprecipitation or immunoaffinity isolation (Figs. 2–4). From now on we will collectively refer to the antigens recognized by mAb 3D9 and 4E8 in the CNS as apl00.

In blots of buccal muscle we observed a faintly stained band at 80 kD (Fig. 1 *B*, lane *M*). At the present time we do not know whether this band is related to the apl00 proteins.

We investigated the membrane association of apl00 by detergent phase partitioning. The 100-kD protein completely partitioned into the detergent phase after extraction with Triton X-114 (Fig. 1 *C*). In addition, the protein was not released from CNS membranes upon treatment with 100 mM Na_2CO_3 (pH 11.5), 100 mM glycine (pH 2.8), 3 M KCl, or 10 mM DTT (data not shown). Taken together these observations suggest that the protein is anchored in the membrane by a transmembrane anchor or by a covalent linkage to membrane lipids.

Examination of the subcellular distribution of apl00 by centrifugation in a discontinuous sucrose gradient (Chin et al., 1989) showed that the major band at 100 kD was enriched at the 0.35 M/0.75 M interface (Fig. 1 *D*), the region of the gradient that has been shown to be enriched in synaptosomes. This distribution contrasted sharply with the distribution of the 85-kD antigen recognized by mAb 6E2, another mAb isolated from our library. This antigen was distributed uniformly in the various fractions (Fig. 1 *D*).

mAb 4E8 consistently precipitated two sharp radiolabeled bands of 100 and 150 kD and several bands between 110 and 130 kD (Fig. 2). The 100-kD band was always the most intensely labeled. Bands of variable intensity were observed at molecular masses below 100 kD, but they did not appear to be specific because they were precipitated also by a control mAb (Fig. 2 *A*, *3D11*) or IgG from normal mouse serum (data not shown). The mobility of the bands was the same under reducing and nonreducing conditions.

Upon treatment with two different *N*-glycosidases, *N*-glycanase and Endo-H, the mobility of all bands increased by the same amount (Fig. 2 *B*). The only exception was the band of highest molecular mass (150 kD), which was only partly shifted upon endoglycosidase treatment (Fig. 2 *B*). This may indicate incomplete digestion by the enzymes, or the presence of O-linked sugars. Furthermore, although not visible in this figure, shorter exposure time of this same gel showed that the broad 92.5-kD band seen after deglycosylation was actually composed of two bands. Treatment with neuraminidase did not change the mobility of any of the bands (data not shown).

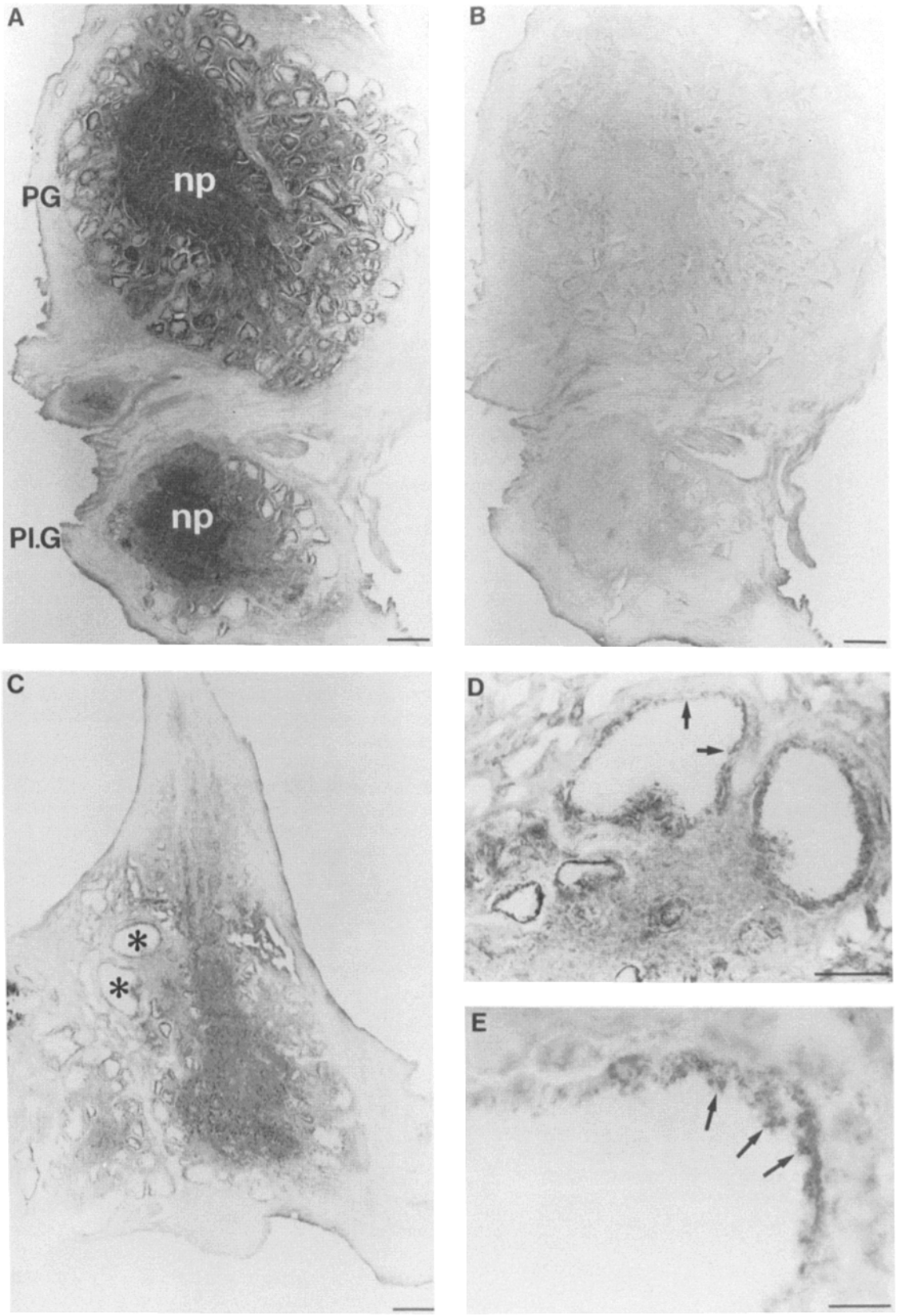

Figure 5. Immunocytochemical distribution of apl00 in sections of *Aplysia* CNS ganglia. (*A*) Pleural ganglion (*Pl.G*) and pedal ganglion (*PG*) stained with mAb 4E8 with an immunoperoxidase technique (purified antibody diluted to 1 μg/ml). Heavy staining is seen in the neuropil (*np*). (*B*) An adjacent section, stained with a control IgG at the same dilution as in *A*. (*C*) Abdominal ganglion, stained with mAb 4E8 (1 μg/ml). Again, the heaviest staining is in the neuropil. The two large neurons labeled with asterisks are shown enlarged in *D*. (*D*) Staining on the surface of two large neurons (*arrows*), as well on other small neurons in the field. (*E*) Immunoreactivity associated with the neuronal surface is on spherical structures, probably representing invaginations of the cell membrane. Bars: (*A–D*) 100 μm; (*E*) 25 μm.

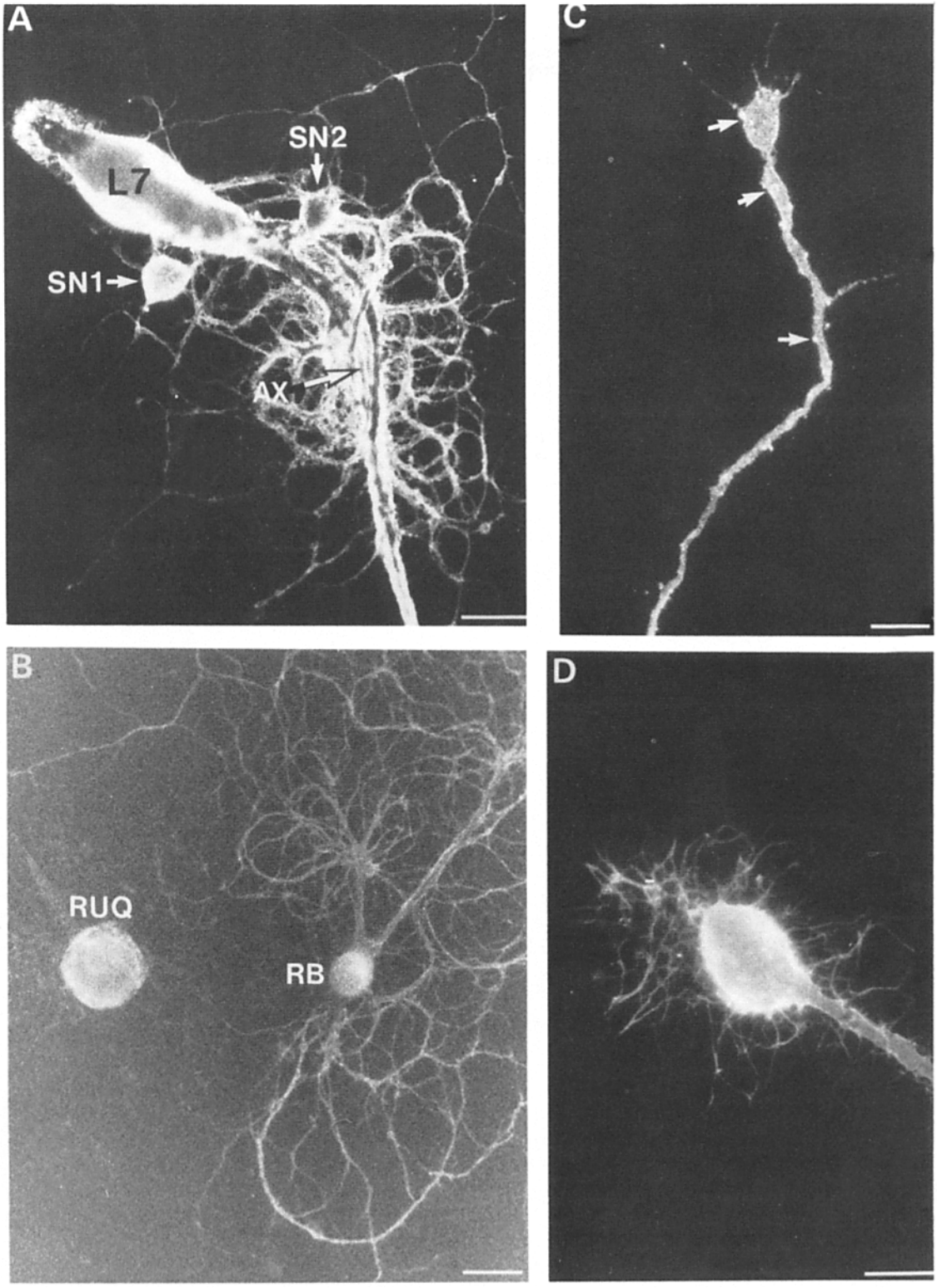

Figure 6. Distribution of ap100 on the surface of cultured, fixed neurons as shown by immunofluorescence. The cells were fixed with paraformaldehyde and stained with mAb 4E8. Bound antibody was revealed by rabbit anti-mouse antiserum, followed by TRITC-labeled goat anti-rabbit antiserum. (*A*) Motor neuron L7 and two sensory neurons (*SN*) placed on each side of the motor neuron. There is heavy staining on the initial axon segment (*AX*), as well as on the fine neurites growing off from the cells. (*B*) The RB cell shows immunofluorescence on its neurites, whereas the neurites of the RUQ cell are unstained. The staining in the cell bodies is partly due to autofluorescence. (*C*) High magnification micrograph of an isolated neurite and associated growth cone. The staining appears to be clustered on the surface of this neurite (*arrows*). Some of the clusters appear to be associated with the points where filopodia bud off from the lamellipodium, and also with fine processes budding off from the neurite. (*D*) High magnification view of a large growth cone and associated filopodia. Immunofluorescence is clearly associated with the plasma membrane. Bars: (*A* and *B*) 100 μm; (*C* and *D*) 10 μm.

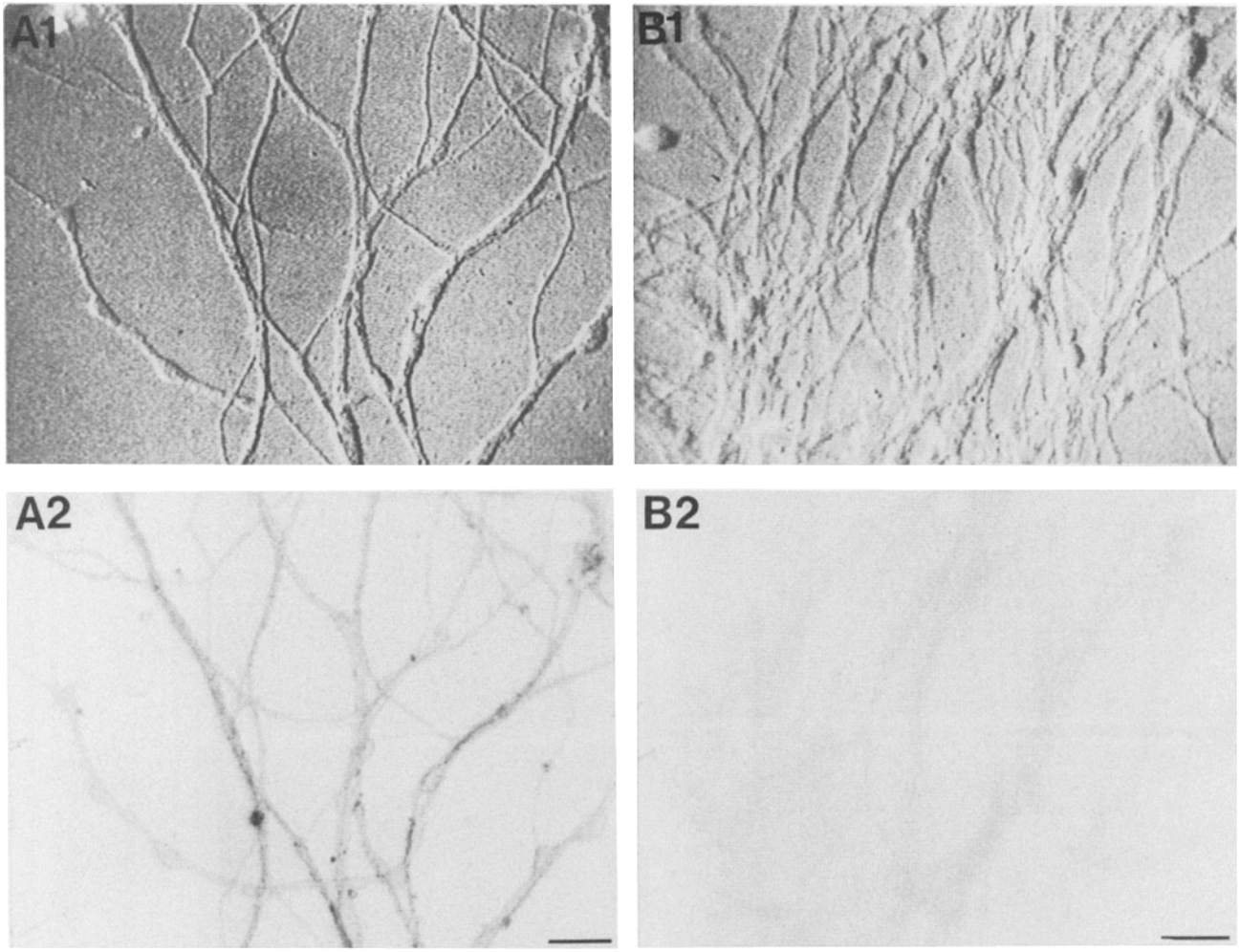

Figure 7. Purified apl00 blocks staining of neurites by mAb 4E8. In this and the following micrographs the VCR-stored fluorescent images have been printed with black and white reversed to enhance the contrast. (*A*) Neurites of a LUQ cell in culture for 2 d are stained with 10 μg/ml FITC-labeled 4E8 (*A1*, Nomarski; *A2*, fluorescence). (*B*) Neurites of a companion LUQ cell in culture for 2 d that was treated with 10 μg/ml FITC-labeled 4E8 preincubated with 100 μg/ml purified apl00 (*B1*, Nomarski; *B2*, fluorescence). Note that the level of staining of the neurites is significantly reduced by preincubating the antibody with the purified protein. Bars, 20 μm.

mAb 3D9 appeared to recognize the same molecular species as mAb 4E8. Preabsorption of CNS extract with mAb 4E8 strongly reduced the amount of radiolabeled proteins precipitated by mAb 3D9 (Fig. 3 *A*). Furthermore, all molecular species eluted from a 4E8 affinity column cross-reacted with the 3D9 antibody (Fig. 3 *B*). Nevertheless, we cannot exclude that both mAbs might have different affinities for the different isoforms of apl00. On the other hand, mAbs 4E8 and 3D9 appeared to recognize different epitopes on the same protein, since binding of FITC-labeled 4E8 mAb to the cell surface was not inhibited by unlabeled 3D9 mAb, and vice versa (results not shown).

The proteins could be isolated almost to homogeneity by immunoaffinity chromatography (Fig. 4, lanes *4* and *5*). Disregarding possible losses during purification, the abundance of the apl00 proteins can be calculated to be 0.3–0.5% of total CNS protein, or 1% of total CNS membrane protein.

Expression of apl00 in Neurons In Situ and in Culture

The neuropil of all CNS ganglia was stained intensely with the mAbs. Fig. 5, *A* and *C*, shows the staining of the neuropil by mAb 4E8 in the pleural-pedal ganglion and in the abdominal ganglion, respectively. A similar staining pattern was observed with mAb 3D9 (data not shown). The sheath surrounding the ganglia showed only background staining. In the neuropil, especially if immunostaining was performed in the presence of saponin, the surface of spherical structures, possibly synaptic endings, was heavily stained (data not shown). At higher magnification heavy staining was seen to be restricted to the surface of neurons (Fig. 5 *D*) and appeared to be present on spherical structures on the cell surface (Fig. 5 *E*). The cytoplasm and nuclei of the neurons were free of staining (Fig. 5, *D* and *E*). When the mAb was substituted by nonimmune mouse IgG, the ganglia exhibited a background level of staining (Fig. 5 *B*).

In fixed neuronal cultures immunofluorescence was present on the surface of the cell bodies, neurites, growth cones, and associated filopodia (Fig. 6). There was no obvious difference in the level of expression among different neuronal cell types. The only notable exception encountered so far are the RUQ cells of the abdominal ganglion whose neurites and

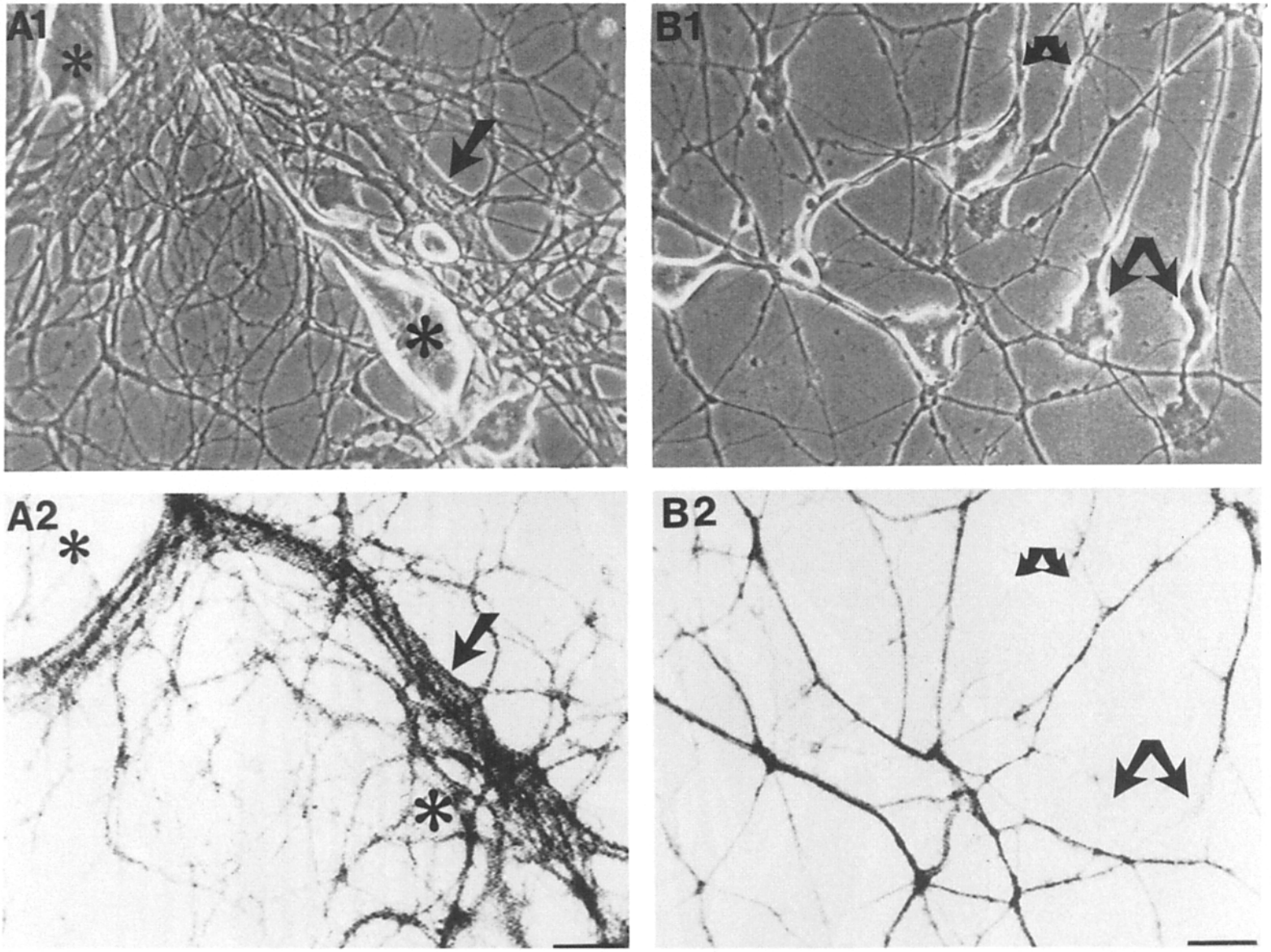

Figure 8. Ap100 is expressed on neurites of living LUQ cells but not RUQ cells. (*A*) Immunofluorescence of FITC-labeled 4E8 mAb on proximal axons of LUQ and RUQ cells in coculture for 3 d. The phase contrast light micrograph (*A1*) shows a region containing the proximal portions of axons of both a LUQ cell (*arrow*) and a RUQ cell (*asterisks*) and their regenerated neurites. Epifluorescent view of the same region (*A2*) shows that the LUQ axon has intense labeling (*arrow*), whereas the RUQ axons (*asterisks*) do not. (*B*) FITC-labeled 4E8 mAb stains distal LUQ neurites but not RUQ neurites. The phase contrast light micrograph (*B1*) shows a region containing the small diameter distal LUQ neurites and RUQ neurites (some indicated with arrows) with their characteristic large growth cones. The fluorescent view of the same region (*B2*) shows positively stained LUQ neurites, whereas the RUQ neurites and growth cones have little if any staining. Bars, 10 μm.

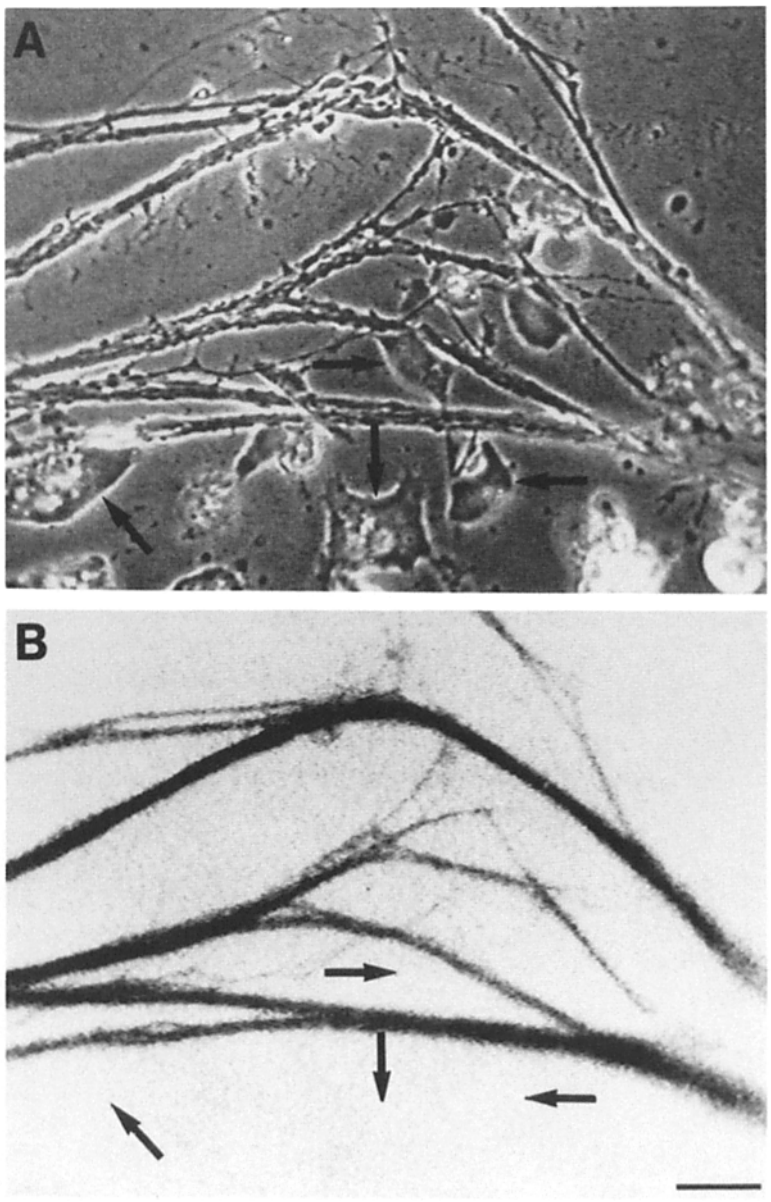

Figure 9. Ap100 is not expressed on surfaces of nonneuronal cells. (*A*) Phase contrast light micrograph of a region containing neurites regenerated from bag cells of the abdominal ganglion in culture for 2 d. Both flattened and spherical nonneuronal cells (some indicated with arrows) isolated along with the bag cells are scattered over the entire area. (*B*) Epifluorescent view of the same region showing the distribution of staining of FITC-labeled 3D9 mAb. Note that the bag cell neurites are heavily stained, whereas the nonneural cells (location of arrows) show little or no staining. Bars, 10 μm.

growth cones showed little or no staining in fixed preparations (Fig. 6 *B*). The staining of the cell body of the RUQ cell in Fig. 6 *B* is due to autofluorescence. In addition to a diffuse type of staining on the surface of the neurites, we observed a clustered type of staining, which was often associated with neuritic buds, branching points, and the insertion of filopodia into the main body of the growth cone (Fig. 6 *C*, *arrows*). The staining in the growth cones reached into the filopodia.

We investigated the pattern of staining of live cells in culture with FITC-labeled mAbs 4E8 and 3D9. The staining of the live cells by FITC-labeled mAb 4E8 (Fig. 7, *A2*) was blocked by pretreating the antibody with the immunoaffinity-purified proteins (Fig. 7, *B2*). After pretreatment of 3D9-FITC with immunoaffinity-purified proteins, a residual staining was still observed on the cell surface. Lack of complete inhibition of 3D9 staining could be due to a lower affinity of the purified proteins for the mAb. The binding of 3D9-FITC and 4E8-FITC was completely inhibited by preincubation of the cells with unlabeled antibody (data not shown).

Consistent with the lack of staining in fixed cultures, the neurites and growth cones of living RUQ cells could not be labeled with the mAbs (Fig. 8).

FITC-labeled 3D9 or 4E8 staining of cocultures of bag cell neurons with their associated nonneuronal cells (fibroblasts, glia, and sheath muscle cells) showed that the neurons alone had positive staining (Fig. 9). Furthermore, the staining appeared to be enriched in areas of synaptic interaction (Glanzman et al., 1989): double staining of sensorimotor cultures with FITC-labeled 4E8 or 3D9 showed that most of the rhodamine-labeled sensory neuron varicosities apposed to the major axons of L7 (Fig. 10 *A*) were stained more intensely than the neighboring regions (Fig. 10 *B*).

4E8 and 3D9 mAbs Perturb Pattern of Axon Growth

Plating individual cells in the presence of 4E8-IgG, 3D9-IgG, or their respective Fab fragments (200 μg/ml) resulted

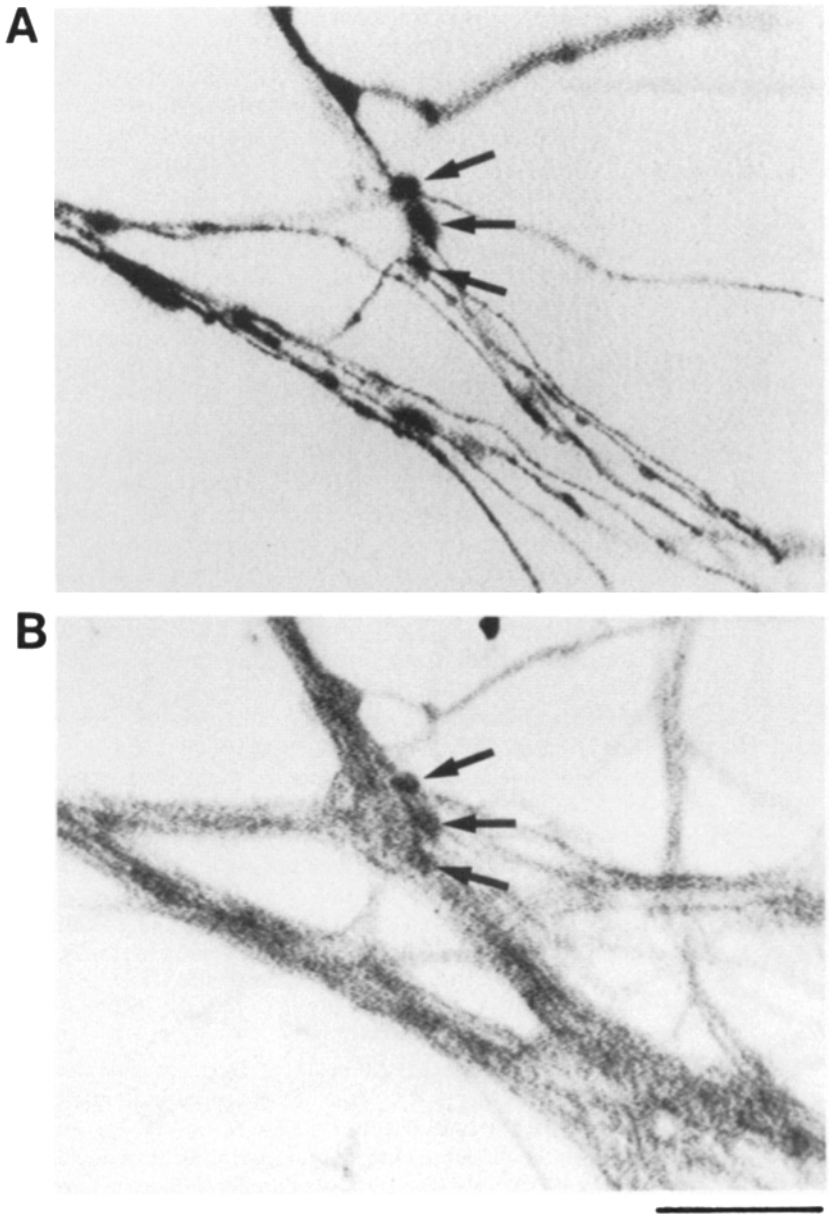

Figure 10. Ap100 appears to be enriched at putative transmitter release sites in sensory neuron L7 cocultures. The sensory neuron evoked a 22-mV excitatory postsynaptic potential in L7. The sensory neuron was injected with lissamine rhodamine and the coculture was thereafter stained with FITC-labeled mAb 4E8. (*A*) Rhodamine epifluorescent view of a portion of the regenerated neurites of a sensory cell that was cocultured with motor cell L7 for 4 d. The fine diameter sensory neuron processes are studded with varicosities or swellings (some are indicated with arrows) and are growing on the thick proximal axons of L7. The labeled sensory cell neurites that are out of focus are located on the surface of the culture dish. (*B*) Fluorescein epifluorescent image of the same region stained with FITC-labeled 4E8 mAb. The axons and neurites of both the motor and sensory cells are stained. Note that the sensory cell varicosities are stained more intensely relative to the major axon of L7 located in the same region. Bar, 10 μm.

in a striking increase in the number of fine diameter neurites emerging from the axon stumps in all neurons treated (36 cells) except the RUQ cells (Fig. 11 *C*), compared with control untreated neurons (26 cells) or neurons exposed to the same concentration of mAb 3D11 (4 cells; Fig. 11 *A*), or to nonimmune mouse IgG (5 cells; Fig. 11 *B*). The neuronal types tested so far with the experimental mAb were LUQ (20 cells), L7 (4 cells), L11 (4 cells), RB (8 cells), and RUQ (6 cells). In addition, the same antibodies bound to the culture dish did not have any effect on neurite fasciculation.

The antibodies appeared to cause the defasciculation of emerging neurites. Acute application of 4E8 (five LUQ cells), 3D9 (four LUQ cells), or their respective Fab fragments (four LUQ cells) on cells extending neurites under control conditions resulted in the rapid (within 5 h) separation of distal neurites such that they now extended as distinct processes (Fig. 12, *D* and *E*). More proximal fasciculated neurites were little affected. In addition, changes in growth cone structure were observed, including the formation of prominent lamellipodia (Fig. 12 *F*). With longer treatment (6–12 h) growth cones of treated cells typically had more and longer filopodial extensions. In the presence of nonimmune mouse IgG (five LUQ cells; Fig. 12, *A–C*) or mAb 3D11 (three LUQ cells; not shown), neurites extended in a fasciculated manner and their growth cones retained their normal rounded morphology.

Discussion

We have identified a group of neuron-specific membrane glycoproteins with molecular masses between 100 and 150 kD, which are expressed in the nervous system of juvenile and adult *Aplysia californica*. We have called this group of proteins apl00. The fact that all proteins are recognized by two different mAbs suggests that the different species are related at the level of the primary amino acid sequence. Mul-

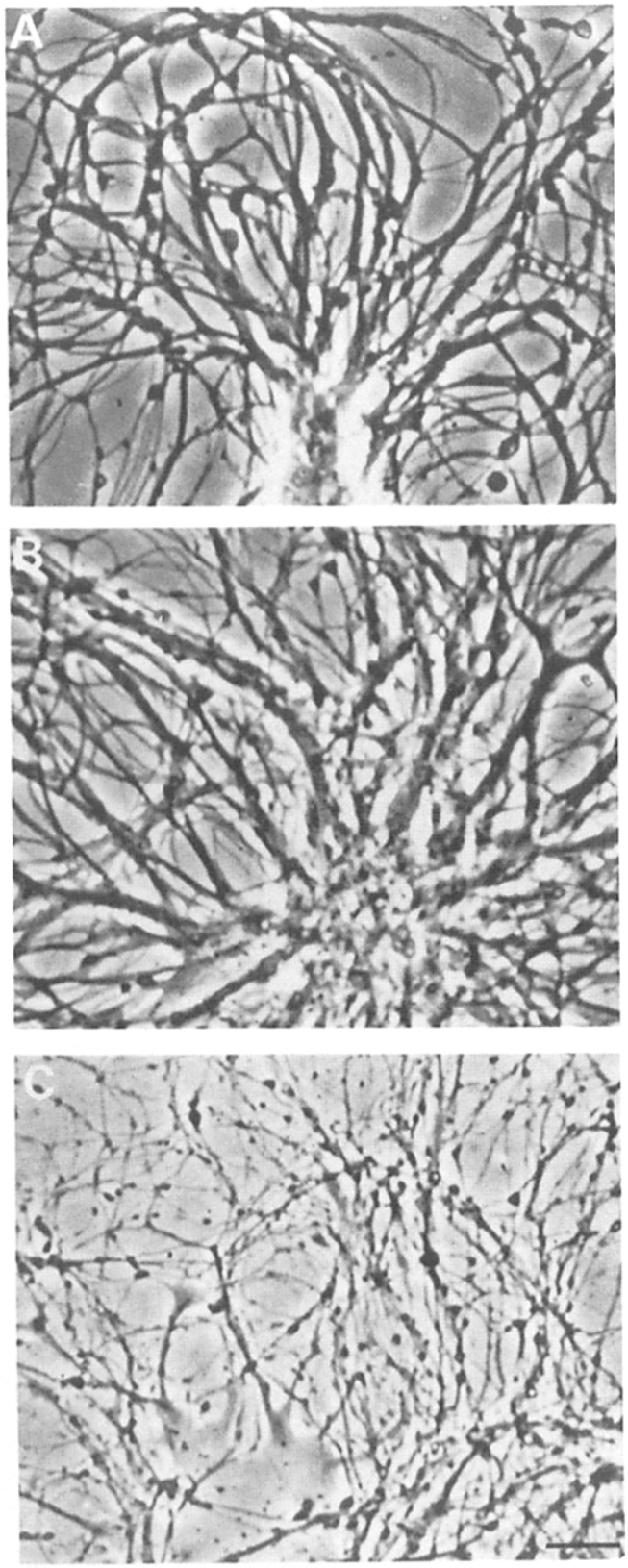

Figure 11. Chronic exposure to anti-apl00 mAbs alters pattern of neurite outgrowth. LUQ neurons were plated in the presence of control mAb 3D11 (*A*), in the presence of control IgG (*B*), or in the presence of 3D9 mAb, and maintained for 2 d in culture. Antibody concentration was 200 μg/ml. Note that the neurites emerging from the distal axon stump of the cell in *A* and *B* are relatively thick and fewer in number compared with those emerging from the axon stump of the cell in *C*, which has many fine diameter neurites. The overall extension of the neurites away from the axon stump was not affected by the 3D9 mAb. Similar results were obtained with 4E8 mAb or with their respective Fab fragments. Bar, 10 μm.

tiple molecular forms have been reported for many developmentally relevant membrane proteins (Jessell, 1988). At this point we cannot exclude the possibility that some of the species are derived from others by proteolysis of higher molecular weight products.

Previous studies on the pattern of neurite growth by *Aplysia* neurons in vitro indicated that neurites of a single identified cell extend as fascicles of finer diameter processes (Schacher and Proshansky, 1983; Ambron et al., 1985; Glanzman et al., 1989). Thus far, all cell types display this pattern of growth with the exception of the RUQ cells (Flaster et al., 1986). The fact that apl00 was present in neurites and growth cones, and that the portion of the molecule containing the epitope(s) recognized by the anti-apl00 mAbs was exposed on the outside of the cell, prompted us to test whether the antibodies would alter the fasciculation of growing neurites. Both whole IgG and Fab fragments of mAbs 4E8 and 3D9 had a dramatic effect on the number of neurites emerging from the axon stumps of individual cells. While neurites of most *Aplysia* neurons in culture under normal conditions (Schacher and Proshansky, 1983) or treated with control antibodies grow in a fasciculated manner, those growing in the presence of either mAb 4E8 or mAb 3D9 were much more defasciculated, as indicated by their fine diameter and increased number. The fact that the same effect was observed with mAbs belonging to two different immunoglobulin subclasses indicates that it is not a simple reaction of the cells to antibodies of a particular subclass. In addition, coating the cell surface with Fab fragments from control mAb 3D11 or nonimmune mouse IgG did not perturb the pattern of growth.

The observations on the RUQ cells of the abdominal ganglion are consistent with the role of these proteins in fasciculation. Growth from the RUQ cells is unaffected by the antibodies, their neurites express very little apl00 proteins, and they grow in a defasciculated manner (Flaster et al., 1986).

The time-lapse observations on actively growing neurites in culture also support the role of these proteins in fasciculation. Whereas control cultures showed only a few growth cones at the tip of growing neurites, cultures treated with the anti-apl00 mAb showed a larger number of flattened growth cones with many filopodia. These effects of the antibodies on growth cones appear to be similar to those reported with antibodies against other cell surface adhesion molecules such as Fasciclin II (Harrelson and Goodman, 1988). Interestingly, the anti-apl00 mAb appeared to cause defasciculation primarily before fascicles were formed. At the light microscope level the anti-apl00 mAb appeared to have little effect on established fascicles proximal to the growing ends. Taken together, these observations suggest that the apl00 proteins are involved in neurite fasciculation.

Defasciculation of presynaptic neuritic bundles is observed at choice points; for example, when axons change their course (Zipser et al., 1989) or when they reach their

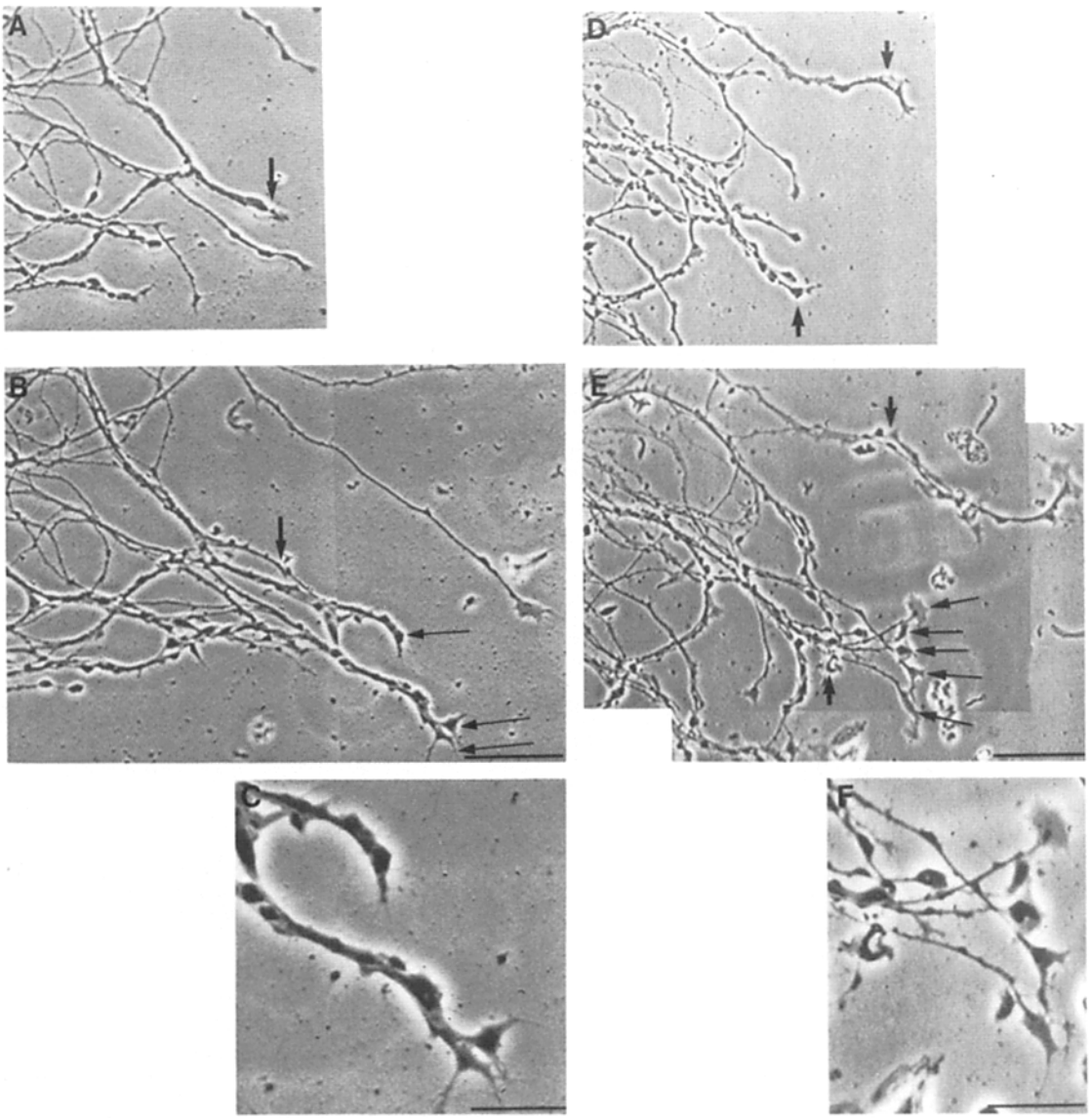

Figure 12. Acute exposure to anti-apl00 mAb causes defasciculation of growing neurites. Phase contrast light micrographs of extending neurites before (*A*, *D*) and after exposure to control IgG (*B*, *C*) and 4E8 mAb (*E*, *F*). Antibody concentration was 200 μg/ml. Bold arrows are reference points. (*B*) Same region of extending neurites from cell in *A* after 6 h in the presence of control IgG. Note that the neurites extending from the reference point (*bold arrow*) have retained their fasciculated pattern. Thin arrows point to leading growth cones. (*C*) Higher magnification view of the leading growth cones of cell in *B*. The bodies of the growth cones have retained their phase dark appearance and relatively narrow lamellipodia. (*E*) Same region of extending neurites from cell in *D* after 6 h in the presence of 4E8 mAb. The neurites extending from the reference points (*bold arrows*) have separated from each other, giving rise to numerous thin processes with leading growth cones (some indicated by the thin arrows). (*F*) Higher magnification view of the leading growth cones of the cell in *E*. Note that the phase dark bodies of most of the growth cones are now surrounded by extended and flattened lamellipodia. Not seen are the numerous fine and elongated filopodial extensions from these growth cones. Also present are trailing growth cones extending individually behind the leading growth cones. By contrast, trailing growth cones in the control treatment (*C*) extend along the processes of the leading growth cones. Bars: (*B* and *E*) 10 μm; (*C* and *F*) 5 μm.

postsynaptic target (Glanzman et al., 1989). Thus, there is the interesting possibility that these processes might require local changes in the expression of fasciculation-promoting proteins.

Specific Fab fragments against the neural cell adhesion molecule N-CAM and against axonal cell adhesion molecules like L1, neurofascin, and others, promote defasciculation of neurites growing on artificial substrates such as collagen and polylysine (reviewed by Rutishauser and Jessell, 1988). Thus, on the basis of distribution, biochemical characteristics, and defasciculation of neurites by anti-apl00 antibodies, apl00 may be a cell adhesion molecule. Adhesion molecules like L1 (Lagenaur and Lemmon, 1987), TAG-1 (Furley et al., 1990), and N-cadherin (Bixgy and Zhang, 1990) are potent substrates for neuritic growth when attached to a nitrocellulose substrate. Therefore, we investigated whether immunoaffinity-purified apl00 promoted neuritic outgrowth when bound to a nitrocellulose substrate. We did not have consistent results with this type of assay. Possible explanations are an insufficient amount of protein

bound to the substrate, or irreversible denaturation of the protein, or loss of a factor required for activity during purification.

The high level of apl00 expression in the adult nervous system of *Aplysia*, especially at sites known to be enriched in synapses—the neuropil of *Aplysia* ganglia in synaptosome preparations and at sensory neuron varicosities apposed to the motor cell (Glanzman et al., 1989)—suggest that these proteins may also play a role in the function of mature synapses. In this respect it may be interesting to point out that our mAb library was generated against a membrane preparation from juvenile *Aplysia* CNS ganglia and screened for mAb staining the neuropil. Recent observations by Cash and Carew (1989) show that there is a striking increase in the volume of the neuropil during the juvenile stage, possibly reflecting the formation of new synapses. These data are consistent with the idea that the apl00 proteins not only play a role in axonogenesis, but might be also relevant for synaptic structure in the adult nervous system of *Aplysia*.

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