

# A Homologue of the Axonally Secreted Protein Axonin-1 Is an Integral Membrane Protein of Nerve Fiber Tracts Involved in Neurite Fasciculation

Markus A. Ruegg, Esther T. Stoeckli, Rainer B. Lanz, Peter Streit,\* and Peter Sonderegger

Institute of Biochemistry, University of Zurich, CH-8057 Zurich; and \*Brain Research Institute, University of Zurich, CH-8029 Zurich, Switzerland

**Abstract.** Axonin-1 is a glycoprotein that is released from axons of cultured neurons (Stoeckli, E. T., P. F. Lemkin, T. B. Kuhn, M. A. Ruegg, M. Heller, and P. Sonderegger. 1989. *Eur. J. Biochem.* 180:249–258). It has recently been purified from the ocular vitreous fluid of the chicken embryo (Ruegg, M. A., E. T. Stoeckli, T. B. Kuhn, M. Heller, R. Zuellig, and P. Sonderegger. 1989. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:55–63). Immunohistochemistry localized axonin-1 prevalently in developing nerve fiber tracts. The presence of anti-axonin-1 Fab fragments during axon growth in vitro resulted in antibody binding to the axonal surfaces and in a marked perturbation of the fasciculation pattern. Hence, a fraction of axonin-1 is associated with axonal membranes and, by operational criteria, qualifies as a cell adhesion molecule. The major proportion of membrane-associated axonin-1 cosolubilized with the integral membrane proteins. By

physico-chemical, immunological, and protein-chemical criteria, the integral membrane form was found to be highly similar to soluble axonin-1. In common with a number of other cell adhesion molecules, both soluble and membrane-bound axonin-1 express the L2/HNK-1 and the L3 epitopes. Radioactive pulse-chase and double-labeling experiments revealed that the released form was not derived from the membrane-bound form by shedding from the membrane surface, but directly secreted from an intracellular pool. Due to its high degree of similarity to the membrane-associated form and the presence of the L2/HNK-1 and L3 epitopes, reported to be ligands in adhesive cell interactions, adhesive properties are postulated for secreted axonin-1. As a soluble adhesive protein, it may function as a regulator of cell adhesion around its most likely site of secretion, the growth cone.

**D**URING neural development, a variety of interactive processes between axons and their cellular and extracellular matrix (ECM)<sup>1</sup> environments have been observed. Over the past decade, cell surface exposed glycoproteins with adhesive properties to other cells or the ECM have become established as one of the dominant molecular factors promoting the elongation of axons and/or directing them to the target area (for a recent review cf. Rutishauser and Jessel, 1988). Proteins secreted from axons during this developmental period have been implicated in a number of tasks subserving these goals. Secreted proteases were suggested to evoke a local weakening of adhesive cell-cell or cell-ECM interactions resulting in a terrain favorable for the intercalation of the advancing growth cone (Krystosek and Seeds, 1981, 1984). A secretory contribution of axons to the local construction or reconstruction of the ECM at the site

of the developing synapse was implied by the observation that the specialized synaptic ECM induced the formation of postsynaptic specializations in the absence of a presynaptic nerve ending (Burden et al., 1979). Such observations have entailed a number of studies aiming to identify and purify the underlying soluble or extracellularly deposited macromolecules. McMahan and collaborators engaged in the purification of a synaptogenic agent found in extracts of the synaptic basal lamina of the *Torpedo* electric organ. On the basis of its capability to induce the aggregation of acetylcholine receptors on cultured myotubes, they isolated a 95/150-kD doublet glycoprotein, which they called agrin (Godfrey et al., 1984; Nitkin et al., 1987). Recent findings suggest that, in the chick nervous system, agrin-like molecules are secreted from motor neurons (Magill-Solc and McMahan, 1988). Kelly and collaborators identified a proteoglycan of synaptic vesicles that is externalized and deposited in the synaptic cleft; because of its proposed function, it was termed "terminal anchorage protein" or TAP-1 (Caroni et al., 1985; Carlson et al., 1986). Pittman (1985) used an ingenious protease detection technique for the identification and characterization of

1. *Abbreviations used in this paper:* Ax-CAM, axon-associated adhesion molecule; CHAPS, 3-(3-cholamidopropyl)dimethyl-ammonio-1-propane sulfonate; DRG, dorsal root ganglia; ECM, extracellular matrix; NILE, nerve growth factor-inducible large external glycoprotein; PIB, protease inhibitor buffer.

proteases in electrophoretically resolved protein mixtures and found a plasminogen activator and a calcium-dependent metalloprotease among the proteins released from axons of cultured neurons.

In our laboratory, we have chosen the compartmentalized cell culture system devised by Campenot (1977) for the identification of axonally secreted proteins. Two proteins released from axons of embryonic chicken dorsal root ganglion neurons in culture were identified (Stoeckli et al., 1989). One of them, termed axonin-1, was detected in the vitreous humor of the eye and subsequently purified (Ruegg et al., 1989). Axonin-1 was found to be predominantly expressed during the second half of embryonic life, concentrations in the adult being ~20 times lower. To determine the functional role of axonin-1, its expression in the embryonic nervous system was studied by immunohistochemical techniques, and perturbation studies with antibodies were carried out on axons extending from cultured dorsal root ganglia. The data reported here identify and characterize a membrane-bound homologue of axonin-1 and demonstrate its prevalence in nerve fiber tracts. Fab fragments of anti-axonin-1 antibodies added to growing axons bind to the axonal surface and interfere with the formation of axon fascicles. Based on these observations, the membrane-bound form of axonin-1 operationally qualifies as a cell adhesion molecule. The high degree of structural similarity with membrane-bound axonin-1 and the observation that it has structural features in common with established cell adhesion molecules suggests that secreted axonin-1 could have adhesive properties. Hence, it could represent a regulatory element in cell adhesion during neurogenesis.

## Materials and Methods

### Antibodies and Fab Fragments

Antibodies against axonin-1 were raised in rabbit and goat as previously described (Ruegg et al., 1989). IgG of rabbit immune or nonimmune sera were precipitated with 40% ammonium sulfate at 4°C and further purified by anion exchange chromatography (Fast Flow Q; Pharmacia Fine Chemicals, Uppsala, Sweden). The purity of the isolated fractions were checked by SDS-PAGE. Fab fragments were produced by mercuripapain digestion according to Porter (1959). Fab fragments were isolated by cation exchange chromatography (mono S; Pharmacia Fine Chemicals). For antibody perturbation experiments, Fab fragments were concentrated by ultrafiltration (diaflo, PM-30; Amicon Corporation, Danvers, MA) and dialyzed against Dulbecco's PBS.

### Immunohistochemistry

For indirect immunofluorescence localization of axonin-1, 12- $\mu$ m-thick frozen sections were cut from 5.5-d embryonic spinal cord and 8-d retina (stage 28 and stage 34, respectively; Hamburger and Hamilton, 1951), fixed in a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4. Goat antiserum against axonin-1 was added at a dilution of 1:1,000 and incubated for 14 h at 4°C. As a second antibody, FITC-conjugated rabbit anti-goat IgG (Zymed Laboratories Inc., San Francisco, CA) was added at a dilution of 1:20 and incubated for 90 min at 21°C. After staining, the sections were coverslipped with 90% glycerol, 10% PBS, pH 8.0, containing 1 mg/ml phenylenediamine, and examined with a fluorescence microscope (Wild and Leitz AG, Zurich, Switzerland) using the L2 band pass filter set for FITC.

### Cell Cultures

Dissociated cells from dorsal root ganglia of 10-d chicken embryos were cultivated on collagen-coated tissue culture dishes (35 mm in diameter) as previously described (Sonderegger et al., 1984). Proliferation of non-

neuronal cells was minimized by the inclusion of 0.12 mM 5-fluorodeoxyuridine and 0.3 mM uridine (both from Sigma Chemical Co., St. Louis, MO). For pulse-labeling and pulse-chase experiments, a density of 350,000–400,000 cells per dish was found to be most suitable.

For antibody-induced perturbation of neurite fasciculation, dorsal root ganglia were explanted into small droplets of medium containing immune or nonimmune Fab fragments at a concentration of 100  $\mu$ g/ml on a polylysine-coated culture dish. After 1 d, when the ganglia had attached, 1 ml of culture medium containing immune or nonimmune Fab fragments was added. After 2–3 d in vitro the cultures were washed twice with Dulbecco's PBS and fixed with 2.5% (vol/vol) glutaraldehyde in PBS for 30 min at room temperature. The growth pattern of the axons was examined with an inverted microscope, equipped with phase contrast optics (diaphot; Nikon, Japan).

To localize the added Fab fragments at the electronmicroscopic level, dorsal root ganglia (DRG) were grown on polylysine-coated cover slips (thermanox, Lux, Division of Miles Laboratories Inc., Naperville, IL). After 2–3 d, the DRG were washed extensively and fixed with 2.5% glutaraldehyde in 0.12 M sodium phosphate, pH 7.3, for 30 min at room temperature, rinsed twice with the same buffer, and incubated for 30 min in a blocking solution containing 20 mM lysine in 0.1 M sodium phosphate, pH 7.3. After incubation with 0.3% gelatin (type A from porcine skin; Sigma Chemical Co.), 10% horse serum (Amimed AG, Basel, Switzerland) in 0.12 M sodium phosphate, pH 7.3 (incubation buffer) for 30 min, peroxidase-conjugated goat anti-rabbit IgG (Bio-science-products AG, Emmenbrücke, Switzerland) was diluted to a final concentration of 24  $\mu$ g/ml and incubated overnight at room temperature. Cultures were washed extensively with the incubation buffer (3  $\times$  20 min), followed by three washes with 0.12 M sodium phosphate, pH 7.3. Peroxidase activity was localized by reaction with freshly prepared 0.05% (wt/vol) 3,3'-diaminobenzidine-4HCl (Serva Fine Biochemicals, Heidelberg, FRG), 0.01% H<sub>2</sub>O<sub>2</sub> in 100 mM NaCl, 50 mM Tris-HCl, pH 7.3. Cultures were postfixed with 1% osmiumtetroxide (Serva Fine Biochemicals) for 1 h and treated with 1% uranyl acetate (Merck, Darmstadt, FRG) in 50 mM sodium acetate, pH 5 for 2 h at 4°C. Cultures were dehydrated and embedded in Epon 812 (Fluka, Buchs, Switzerland).

### Metabolic Labeling of Cell-bound and Released Axonin-1 of Cultured DRG Neurons

Proteins were metabolically labeled in the presence of 5% horse serum and 5% chicken embryo extract. For long term labeling, [<sup>3</sup>H]leucine proved less harmful to the cells than [<sup>35</sup>S]methionine. The medium for [<sup>3</sup>H]leucine labeling was composed of leucine-free growth medium substituted with 100  $\mu$ M unlabeled leucine and 0.2 to 0.25 mCi/ml [<sup>3</sup>H]leucine. [<sup>3</sup>H]leucine, purchased at 1 mCi/ml (New England Nuclear, Boston, MA), was dried by vacuum centrifugation (Speed Vac concentrator; Savant, Farmingdale, NY) in the presence of a 1/10 vol of MEM (without sodium bicarbonate and L-leucine) and redissolved in a 1/10 vol of H<sub>2</sub>O. The osmolarity of this medium was 325  $\pm$  10 mosM. The concentrated [<sup>3</sup>H]leucine was stored at -70°C until required. For [<sup>35</sup>S]methionine labeling, the growth medium contained 25  $\mu$ M unlabeled amino acid and 0.2–0.25 mCi/ml [<sup>35</sup>S]methionine (New England Nuclear).

Conditioned media were collected and the cells were washed three times with 2 ml of fresh medium. The medium of the first wash step was added to the conditioned medium. All solutions were buffered by the addition of 1/10 vol of 200 mM Hepes, pH 7.2. For the immunoprecipitation of metabolically labeled axonin-1, 500  $\mu$ l of vitreous fluid containing ~1–2  $\mu$ g of unlabeled axonin-1, were added to all conditioned media as a carrier. The volume was adjusted to 4.5 ml and the samples were centrifuged at 100,000 g for 1 h (50 Ti; Beckman Instruments, Inc., Palo Alto, CA; 40,000 rpm) to remove cells and cell debris. For immunoprecipitation, the medium was adjusted to 150 mM NaCl, 5 mM EDTA, 1% (wt/vol) Triton X-100, 50 mM Tris-HCl, pH 7.5 by the addition of a 10-fold concentrated stock solution, and 0.1 ml of rabbit anti-axonin-1 antiserum was added. After incubation overnight at room temperature, 100  $\mu$ l of a suspension (1 vol/9 vol) of glutaraldehyde-fixed *Staphylococcus aureus* in 150 mM NaCl, 5 mM EDTA, 1% (wt/vol) Triton X-100, 50 mM Tris-HCl, pH 7.5 was added, and incubation continued for 2 h at room temperature. Bound antigen-antibody complexes were released by boiling for 5 min in 60  $\mu$ l of SDS-PAGE sample buffer.

For the isolation of cell-bound axonin-1, the washed cells were solubilized by the addition of 1 ml of a solution containing 1% (wt/vol) SDS, 25 mM EDTA, 50% (vol/vol) vitreous fluid in Dulbecco's PBS (Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free), preheated to 90°C in a water-bath. Undissolved material was collected with a rubber policeman. The pooled samples were sonicated for 15 min and subsequently heated in a boiling water bath for 3 min. To reduce SDS to a con-

centration compatible with antigen-antibody complex formation, the samples were diluted with 4 vol of 2% NP-40, 25 mM EDTA, 0.3 M NaCl, 10 mM sodium phosphate, pH 7.4, and shaken gently for 1 h at room temperature. After centrifugation for 1 h at 100,000 g (50 Ti; Beckman Instruments, Inc., 40,000 rpm), the immunoprecipitation was carried out as described above, except that the volumes of the antisera and *Staphylococcus aureus* suspensions were twice that in the above procedure.

The immunoprecipitated proteins were separated by SDS-PAGE on 7.5% gels. To locate axonin-1, the gels were quickly stained with Coomassie brilliant blue (Serva Fine Chemicals) and a fluorographic replica was made to check for the purity of the immunoprecipitation. Fluorography was carried out according to the manufacturer's recommendations, using a commercially available enhancer (En<sup>3</sup>Hance; New England Nuclear), and exposure to medical x-ray films (Fuji, Japan) was for 3 d at -70°C. Protein bands corresponding to axonin-1 were cut out from the gels, rehydrated, and incubated for 2 d at room temperature in a mixture of 10 vol of Lipuluma (Lumac/3M, Schaesberg, The Netherlands), 1 vol of Lumasolve (Lumac/3M), and 0.2 vol of water and counted in a liquid scintillation counter (Kontron, Zurich, Switzerland).

### **External Biotin Labeling of Cultured DRG Neurons and Affinity Chromatography on Streptavidin-Agarose**

Externally accessible proteins of intact DRG neurons in dissociated culture were covalently modified with the amino group-specific reagent biotin-X-NHS (Calbiochem-Behring Corp., San Diego, CA) (Hare and Lee, 1989; Boxberg, 1987). The conditioned medium was removed, and the cells were washed three times with biotinylation buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl<sub>2</sub>, 1.4 mM CaCl<sub>2</sub> in 26 mM sodium bicarbonate buffer, pH 7.2). The osmolarity was adjusted to 325 mosM by the addition of D-glucose. A stock-solution of 3 mg/ml biotin-X-NHS in water-free DMSO was diluted to a final concentration of 30 µg/ml in biotinylation buffer. Incubation with the labeling reagent was for 2 min at 37°C. The reaction was stopped by removal of the labeling medium and the addition of 2 ml MEM.

Biotinylated cells were washed twice with PBS and dissolved in 0.5 ml 1% (wt/vol) SDS, 5 mM EDTA, 50% (vol/vol) vitreous fluid in Dulbecco's PBS (Ca<sup>2+</sup>-, Mg<sup>2+</sup>- free), preheated to 90°C. The tissue culture dishes were rinsed with an additional 0.5 ml of 1.1% NP-40, 5 mM EDTA, 50% vitreous fluid in Dulbecco's PBS (Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free). The samples were pooled, sonicated, heated for 3 min in a boiling water bath, and stored at -70°C until required.

Biotinylated proteins were isolated by affinity chromatography on Streptavidin-agarose. The solubilized cellular proteins were centrifuged at 100,000 g for 1 h and subsequently incubated for 16 h at room temperature with 100 µl of a suspension of Streptavidin-agarose (Sigma Chemical Co.). After centrifugation, removal of the supernatant (unbound fraction), and extensive washing of the gel with 150 mM NaCl, 5 mM EDTA, 1% (wt/vol) Triton X-100, 50 mM Tris-HCl, pH 7.5, biotinylated proteins were eluted with 1 ml of 1% (wt/vol) SDS, 25 mM EDTA, 50% vitreous fluid in Dulbecco's PBS (Ca<sup>2+</sup>-, Mg<sup>2+</sup>- free). To reestablish conditions permissive for antigen-antibody complex formation, 4 ml of 2% (wt/vol) NP-40, 25 mM EDTA, 0.3 M NaCl, 10 mM sodium phosphate, pH 7.4 were added to the eluted material. Both the bound and the unbound fraction were subjected to immunoprecipitation and quantification of the radioactivity incorporated into axonin-1, as described above.

As a control for the protection of intracellular proteins, biotinylation of cytosolic aspartate aminotransferase was assessed using the same protocol as for axonin-1. Polyclonal rabbit antiserum against cytosolic aspartate aminotransferase and pure chicken cytosolic aspartate aminotransferase, used as a carrier, were provided by Dr. H. Gehring.

### **Preparation and Subfractionation of Brain Membranes**

Brain membranes from 10-14-d-old chicken embryos were prepared according to Hoffman et al. (1982). Brains were removed and washed at 4°C in 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4. For homogenization and all the following centrifugation steps, a mixture of protease inhibitors (all from Sigma Chemical Co.) was added to a final concentration of 0.5 mM PMSF, 5 µM pepstatin A, 10 µM leupeptin, and 1% (vol/vol) aprotinin (protease inhibitor buffer, PIB). Tissue disruption was carried out using a Dounce homogenizer with six strokes of the loose and three strokes of the tight pestle. After centrifugation for 15 min at 40,000 g (SS-34; Sorvall Instruments Div., Newton, CT; 20,000 rpm), the volume of the pellet was measured and 2 vol of 2.25 M sucrose in PIB were added. The suspension was placed in centrifuge

tubes and each aliquot was overlaid with 1/3 vol of 0.8 M sucrose. After centrifugation at 100,000 g (T-865; Sorvall Instruments Div., 38,000 rpm) for 1 h, the material at the interface was collected, washed twice, and resuspended in PIB. After determination of the protein concentration, buffer was added to give a final protein concentration of ~25 mg/ml. All procedures were carried out at 4°C; if not used immediately, the membrane suspension was stored at -70°C.

Before membrane subfractionation, soluble axonin-1, sequestered in membrane vesicles, was released by sonication in an ice-bath (Kreibich and Sabatini, 1974) using a sonifier equipped with a microtip (Sonifier 250; Branson Sonic Power Co., Danbury, CT). Sonication was carried out in five periods of 20 pulses (40 watts, 0.5 s) allowing 1-min intervals for cooling. Sonicated membranes were isolated by centrifugation at 100,000 g for 1 h at 4°C. To solubilize peripherally adhered proteins, membranes were consecutively exposed to mild and harsh release conditions. Protein release under mild conditions was in 1 M NaCl in PIB for 90 min at 4°C, followed by centrifugation at 100,000 g for 1 h at 4°C. Release under harsh conditions was at high pH (Steck and Yu, 1973). In using a buffered solution we followed the suggestion of Fujiki et al. (1982), however, 50 mM triethylamine, pH 11.5, was preferred to sodium carbonate (Snow et al., 1988). Incubation in 50 mM triethylamine, pH 11.5, in the presence of the protease inhibitors, was for 90 min at 4°C, and a centrifugation at 100,000 g for 1 h at 4°C followed. The pellet of the stripped membranes was resuspended by the addition of PIB to a final protein concentration between 2-4 mg/ml. Extraction of membrane proteins was carried out by the addition of 1 vol of 2% (wt/vol) 3-(3-cholamidopropyl)dimethyl-ammonio-1-propane sulfonate (CHAPS) in PIB with gentle shaking for 90 min at 4°C. Undissolved material was removed by centrifugation at 100,000 g for 1 h at 4°C and solubilized in hot SDS.

### **Immunoaffinity Chromatography of Axonin-1**

For isolation of the integral membrane form of axonin-1, CHAPS-solubilized proteins of stripped brain membranes were passed through immunoaffinity columns with immobilized rabbit polyclonal anti-axonin-1 IgG (CNBr-activated Sepharose; Pharmacia Fine Chemicals; containing ~5 mg of IgG per milliliter packed gel). After binding, the columns were washed with at least 40 column vol of 1% CHAPS, 300 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4 including the mixture of protease inhibitors. Bound antigen was eluted with 50 mM diethylamine, 0.5% CHAPS, pH 11.5. The eluate was neutralized immediately by the addition of 1/10 vol of 1 M Tris-HCl, pH 7.0. All procedures were carried out at 4°C.

### **Protein Determinations**

Concentrations of soluble protein in the range of 0.2-1.2 mg/ml were determined according to Bradford (1976), using a commercially available product (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, CA). Protein of brain membrane suspensions was quantified using a modification of the Bradford procedure adapted for measurements in the presence of SDS (Rubin and Warren, 1977). Lower protein concentrations were determined after modification with fluorescamine (Fluram; Roche, Basel, Switzerland) as described by Böhlen et al. (1973). BSA was used as a standard.

### **Electrophoretic Procedures**

SDS-PAGE was carried out according to Laemmli (1970). Two-dimensional SDS-PAGE was performed according to O'Farrell (1975) under previously detailed conditions (Sonderregger et al., 1985). For fluorography (Bonner and Laskey, 1974), a commercially available acetic acid-based enhancer (En<sup>3</sup>Hance; New England Nuclear) was used. Silver staining was according to Switzer et al. (1979) as modified by Oakley et al. (1980). Radioactive molecular mass markers were the [<sup>14</sup>C]methylated forms of cytochrome c (12 kD), carbonic anhydrase (30 kD), albumin (69 kD), phosphorylase (97 kD), and myosin (200 kD) (all from New England Nuclear and mitochondrial aspartate aminotransferase (45 kD) alkylated with [<sup>14</sup>C]N-ethylmaleimide; for silver stained gels, cytochrome c (12 kD), carbonic anhydrase (30 kD), ovalbumin (45-46 kD), albumin (69 kD), phosphorylase (97 kD), β-galactosidase (116 kD), myosin (200 kD) (all from Sigma Chemical Co.) were used. In some gels (Fig. 3, A and B), the marker proteins were supplemented with RNA polymerase (Pierce Chemical Co., Rockford, IL) displaying subunit bands at 165, 155, and 39 kD.

### **Peptide Mapping and Deglycosylation**

Peptide mapping of soluble and membrane-associated axonin-1 was carried

out as described by Cleveland (1983). In brief, 5  $\mu$ g of axonin-1 were separated by SDS-PAGE on a 5% gel. After visualization of protein bands by a short staining with Coomassie blue, axonin-1 bands were cut out of the gel and equilibrated in 0.1% (wt/vol) SDS, 1 mM EDTA, 10% (vol/vol) glycerol, 0.3% (vol/vol)  $\beta$ -mercaptoethanol, 125 mM Tris-HCl, pH 6.8 for 1 h. The gel pieces containing axonin-1 were loaded on a SDS-PAGE gradient gel (10–17.5% acrylamide). For partial proteolytic digestion during electrophoresis, endoproteinase Glu-C (EC 3.4.21.19, from *Staphylococcus aureus* V8; Boehringer Mannheim GmbH, FRG) was added at either 0.1% or 0.5% (wt/wt). To enhance proteolysis, electrophoresis was stopped for 10 min when the proteins were most concentrated at the interphase between stacking and separating gel. The resulting peptide fragments were visualized by silver staining.

For the enzymatic deglycosylation with glycopeptidase F (peptide *N*-glycohydrolase F, EC 3.2.2.18, from *Flavobacterium meningosepticum*; Boehringer Mannheim), 3  $\mu$ g of soluble or membrane-bound axonin-1 were precipitated in chloroform/methanol (Wessel and Fluegge, 1984), and redissolved in 1% SDS, 5%  $\beta$ -mercaptoethanol, 50 mM EDTA in 20 mM sodium phosphate, pH 7.2, preheated to 90°C. After dilution of the sample to 0.2% SDS, 1%  $\beta$ -mercaptoethanol, Triton X-100 was added to give a final concentration of 1% (wt/vol) Triton X-100. Deglycosylation was started by the addition of 1 U of glycopeptidase F. Incubation was at 37°C for 24 h.

### Immunoblotting

Proteins were resolved by one- or two-dimensional SDS-PAGE. Electrotransfer onto nitrocellulose (Schleicher and Schuell, Dassel, FRG) was carried out according to Towbin et al. (1979) at 40 V for 16 h and 4°C. Immunodetection of antigens was performed as described by Hawkes et al. (1982). To increase the sensitivity when monoclonal antibodies were used, a modified procedure involving a bridge antibody between the antigen-specific and the peroxidase-conjugated antibody was applied. After incubation of the antigen-specific monoclonal antibodies overnight at 4°C, an affinity-purified antibody against mouse IgG (goat anti-mouse IgG; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was incubated at a concentration of 5  $\mu$ g/ml for 6 h at room temperature. Subsequently, a peroxidase-conjugated antibody against goat IgG (rabbit anti-goat IgG, peroxidase conjugated; Bioscience-products AG) was added and the incubation carried on overnight at 4°C. The peroxidase-reaction was with 0.01% H<sub>2</sub>O<sub>2</sub> (Fluka AG, Buchs, Switzerland), 0.5 mg/ml 4-chloro-naphthol (E. Merck, Darmstadt, FRG), 200 mM NaCl in 50 mM Tris-HCl, pH 7.4. The reaction was stopped by washing the nitrocellulose with distilled water. Antisera against axonin-1, raised in rabbit or goat, were diluted 1/4,000, and monoclonal antibodies from ascitic fluids were diluted 1/1,000. HNK-1 was used as the undiluted culture supernatant of the mouse-mouse hybridoma (ATCC TIB 200; American Type Culture Collection, Rockville, MD). L2 and L3 were used as the undiluted culture supernatants of rat-mouse hybridomas (provided by Dr. M. Schachner). Polyclonal rabbit IgG against N-CAM and Ng-CAM (provided by Dr. G. M. Edelman and coworkers) were used at a concentration of 20  $\mu$ g/ml. Polyclonal IgG against G4, F11, and neurofascin (provided by Dr. F. Rathjen and coworkers) were used at a concentration of 25  $\mu$ g/ml.

### Ouchterlony Double Diffusion Test

For the Ouchterlony double diffusion-in-gel technique (Nilsson, 1983), 1.5% agarose in PBS was heated to 95°C and poured onto 20  $\times$  65-mm glass slides to a thickness of 3 mm. After congelation, the coated slides were stored in a humid chamber at 4°C. Immediately before use, 3-mm-wide circular basins were punched out and filled with antigen or antiserum solutions. Incubation was for 48 h at 4°C.

### ELISA

ELISA was performed as previously described (Ruegg et al., 1989). All measurements were carried out in 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, or 1% CHAPS, 50 mM Tris-HCl, pH 7.5. The concentration of axonin-1 used for the calibration curves of ELISAs was determined by measurement of the absorption at 220 nm ( $\epsilon_{mM} = 1,850$ , as determined by amino acid analysis). The results obtained by ELISA were in good agreement with the estimations based on immunoprecipitation, followed by SDS-PAGE and silver staining, where the relative intensity of axonin-1 was determined by comparison with a dilution series of a known quantity of pure axonin-1.

## Results

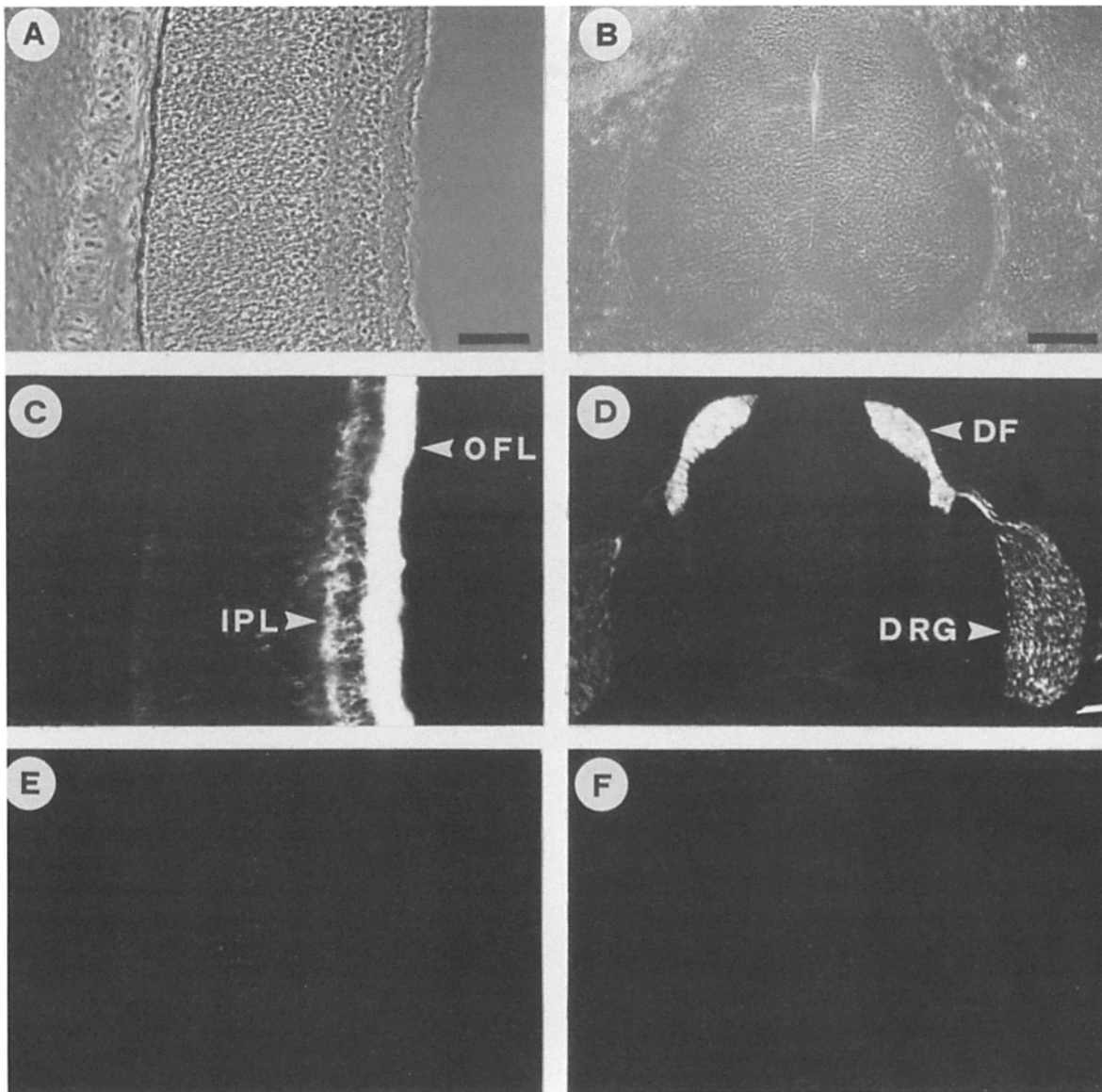
### Axonin-1-like Immunoreactivity Is Associated with Developing Nerve Fiber Tracts

To localize axonin-1 in developing central nervous tissue, cryostat sections of aldehyde-fixed tissue were subjected to indirect immunofluorescence staining. In 5.5-d embryonic chicken retina, the optic fiber layer was intensely stained, weaker staining of diffuse appearance was found in the developing inner plexiform layer (Fig. 1, A and C). In 8-d embryonic spinal cord, axonin-1-like immunoreactivity was most intense in the dorsal funiculus, weaker staining occurred in the dorsal roots and the dorsal root ganglia (Fig. 1, B and D). No fluorescence was detected with preimmune serum used at the same dilution (Fig. 1, E and F). With monoclonal antibodies against axonin-1, the same staining pattern was obtained (not shown), hence, a specific axonin-1-like immunoreactivity was involved.

Predominant association with nerve fiber tracts is typically found with a group of adhesive glycoproteins, termed axon-associated adhesion molecules (AxCAMs), because of their topographic restriction to axonal membranes of nerve fiber tracts (Rutishauser and Jessel, 1988). In the retina, the staining pattern of axonin-1 was virtually identical to that reported for Ng-CAM (Thiery et al., 1985; Daniloff et al., 1986), G4 (Rathjen et al., 1987a), and 8D9 (Lemmon and McLoon, 1986), whereas the reported immunoreactivity of neurofascin is distinct from axonin-1 by its confinement to the innermost part of the optic fiber layer (Rathjen et al., 1987b). In the spinal cord at embryonic day 5 or 6, the immunoreactivity for axonin-1 is virtually restricted to the dorsal funiculus, whereas the reported immunoreactivity pattern for Ng-CAM (Thiery et al., 1985; Daniloff et al., 1986) and G4 (Rathjen et al., 1987b) also includes the white matter of the ventral horn. The distribution of neurofascin in the spinal cord was reported to be even more widespread, including the dorsal and ventral funiculus and the ventral commissure (Rathjen et al., 1987b).

### Fab Fragments of Anti-Axonin-1 Antibodies Bind to Growing Axons and Perturb Their Fasciculation

As a first step in the investigation on a functional role of axonin-1 in cell adhesion, an antibody perturbation assay of axon fasciculation was chosen. The specificity of the polyclonal rabbit antibodies used has been documented under a variety of conditions (Ruegg et al., 1989; this paper); of particular pertinence, no reactivity to proteins in the molecular weight range of the chicken cell adhesion molecules involved in axon fasciculation was detectable with these antibodies by immunoaffinity chromatography of brain membrane proteins under native conditions (see Fig. 3 A, lane I). In all of eight independent perturbation experiments, the presence of anti-axonin-1 Fab fragments in the medium of dorsal root ganglia cultures during the period of axon extension had a dramatic impact on the axonal growth pattern (Fig. 2). Axons grown in the absence of antibodies formed fascicles with obvious radial orientation (Fig. 2 B). The presence of Fab fragments during axon elongation resulted in a fine meshwork of relatively thin fascicles (Fig. 2 A); most notably, the radial orientation of the axon fascicles, observed un-



**Figure 1.** Immunohistochemistry localizes axonin-1 in nerve fiber tracts. Aldehyde-fixed frozen sections of 8-d-old embryonic retina (*A*, *C*, and *E*) and 5.5 d-old embryonic spinal cord (*B*, *D*, and *F*) were subjected to indirect immunofluorescence staining using anti-axonin-1 antiserum and FITC-conjugated second antibodies. *A* and *B*, phase optics. *C* and *D*, anti-axonin-1 antiserum. The arrows in *C* indicate the optic fiber layer (*OFL*) and the inner plexiform layer (*IPL*), respectively. The arrows in *D* indicate the dorsal funiculus (*DF*) and the dorsal root ganglion (*DRG*), respectively. *E* and *F*, controls with preimmune serum at the same dilution as immune serum. Bars: (*A*, *C*, and *E*) 60  $\mu\text{m}$ ; (*B*, *D*, and *F*) 150  $\mu\text{m}$ .

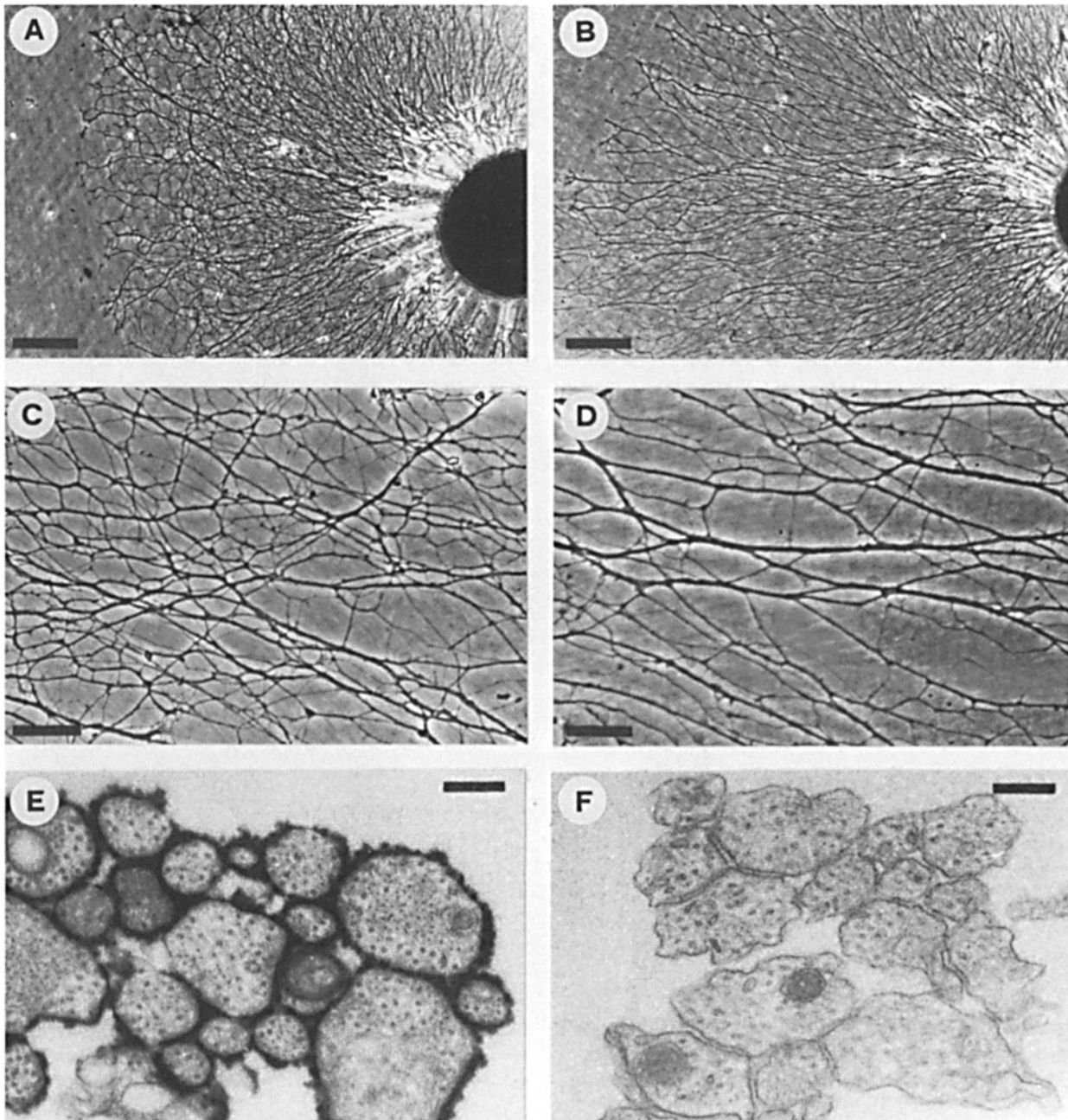
der standard culture conditions, had virtually disappeared. At higher magnification (Fig. 2, *C* and *D*), a markedly finer texture of the axonal meshwork (Fig. 2 *C*) was the most obvious effect observed when axons were grown in the presence of anti-axonin-1 Fab fragments. A reduction of the fascicle diameter was less striking, yet clearly reproducible. If thin fascicles at the periphery of the ganglia were inspected, an increased tendency of single fibers to branch off their fascicle was obvious.

The ultrastructural location at the end of a perturbation experiment of the added Fab fragments was determined by immunoelectron microscopy using a peroxidase-conjugated

second antibody (Fig. 2, *E* and *F*). Intense staining of axonal membranes in axon fascicles was found (Fig. 2 *E*), suggesting a membrane-bound form of axonin-1 as the site of action in perturbing fasciculation.

#### ***Membrane-associated Axonin-1 Comprises a Major Integral Membrane Protein and a Minor Peripheral Membrane Component***

The nature of membrane association of axonin-1 was investigated on sonicated brain membranes prepared from 14-d-old chick embryos by consecutive exposure to selected re-



**Figure 2.** Anti-axonin-1 Fab fragments bind to axonal membranes and perturb neurite fasciculation. Dorsal root ganglia were cultured in the presence of 100  $\mu\text{g}/\text{ml}$  of anti-axonin-1 Fab fragments (*A*, *C*, and *E*) or Fab fragments isolated from nonimmune sera, respectively (*B*, *D*, and *F*). After 2 d in culture, cells were fixed with 2.5% glutaraldehyde and the growth pattern of axons was examined on an inverted microscope, equipped with phase contrast optics. *A* and *C*, axon growth pattern obtained in the presence of Fab fragments against axonin-1. Note the virtual absence of a clear radial orientation (*A*) and the fine texture of the axonal meshwork (*C*). *B* and *D*, axon growth pattern in the presence of nonimmune Fab fragments. Note the thicker fascicles with predominantly radial orientation (*B*) and the coarse texture of the axonal meshwork (*D*). *E* and *F*, immunoelectron microscopic localization of Fab fragments at the end of a perturbation experiment. *E*, axons grown in the presence of anti-axonin-1 Fab fragments. *F*, axons grown in the presence of nonimmune Fab fragments. Bars: (*A* and *B*) 200  $\mu\text{m}$ ; (*C* and *D*) 40  $\mu\text{m}$ ; (*E* and *F*) 0.25  $\mu\text{m}$ .

lease conditions (Table I). The high-salt and the high-pH procedures together removed 37% of the axonin-1. About two-thirds of the membrane-bound axonin-1 were refractory to extraction by high salt and high pH, but were solubilized in the presence of CHAPS, and hence, by operational definition, are integral membrane protein. No axonin-1 was found

among the remaining proteins that could only be solubilized by hot SDS.

The highest specific concentration of axonin-1 was determined in the CHAPS-extractable fraction. Specific concentrations in the high-salt and high pH-extracted fractions were lower by a factor of five and twelve, respectively. Obviously,

**Table I. Subfractionation of Brain Membrane-associated Axonin-1**

Solubilization	Protein	Axonin-1		Specific concentration of
	in supernatant	in supernatant		axonin-1 in supernatant
	mg	μg	%	ng/mg protein
Total	907	73	100	—
1 M NaCl	91	9	12	99
pH 11.5	425	18	25	42
1% CHAPS	91	46	63	506
1% SDS	300	<0.1	<0.1	<0.3

Brain membranes from 14-d-old chicken embryos were resuspended at a protein concentration of 27 mg/ml. Soluble axonin-1 enclosed in membrane vesicles (14 μg) was released by sonication. The sonicated membranes were consecutively exposed to 1 M sodium chloride, to 50 mM triethylamine, pH 11.5, to 1% CHAPS, and to 1% SDS. After each step of this sequence, membranes were centrifuged for 1 h at 100,000 g at 4°C and the concentration of protein and axonin-1 determined in the supernatant; the pellet was resuspended in the appropriate solution for the next step. Quantification of axonin-1 was performed with ELISA, except for the SDS fraction, where immunoprecipitations using polyclonal rabbit anti-axonin-1 antibodies and glutaraldehyde-fixed *Staphylococcus aureus* were carried out after dilution to 0.2% SDS and the addition of Triton X-100 to a sixfold excess (wt/vol) over SDS. Precipitates were subjected to SDS-PAGE and silver staining, and the bands corresponding to axonin-1 were quantified with a series of purified axonin-1 standards. The same immunoprecipitation procedure was also used for a validation of the ELISA data. The proportions of axonin-1 in the vesicular, peripheral membrane, and integral membrane protein fractions were in close agreement with those determined by ELISA.

the surface-bound and the integral membrane proteins are populations of markedly distinct composition. Furthermore, the 12-fold higher specific concentration of axonin-1 in the integral membrane protein fraction as compared with the high pH-released proteins clearly indicates that axonin-1 found in the integral membrane fraction does not represent a contamination due to incomplete removal of the surface adsorbed proteins.

#### ***Axonin-1 of the Integral Membrane Protein Fraction Is Highly Similar, Yet Not Identical, to Soluble Axonin-1***

The high-salt and high pH-resistant form of axonin-1 extracted from stripped membranes by 1% CHAPS in the presence of protein inhibitors migrated slightly faster in SDS-PAGE on a 7.5% gel and its relative molecular mass was estimated to be 128 kD, i.e., ~2 kD lower than the relative molecular mass of its soluble counterpart (Fig. 3 A). Digestion with glycopeptidase F resulted in a reduction of the apparent molecular mass by ~15% in both soluble and membrane-bound axonin-1 (Fig. 3 B). The estimated relative molecular mass of deglycosylated membrane-bound axonin-1 was 115 kD, that of the soluble form ~117 kD. Hence, a molecular mass difference of 2 kD was also present after enzymatic removal of N-linked carbohydrate.

Several pieces of evidence indicate that the soluble and the integral membrane forms of axonin-1 are highly similar proteins. (a) A comparison by means of the Ouchterlony double-diffusion test revealed immunological identity, as indicated by the complete fusion of the precipitin lines (Fig. 3 C). (b) Soluble and membrane-bound axonin-1 are also indistinguishable with respect to the number and the pI of their isoelectric variants, as revealed by two-dimensional SDS-PAGE (not shown). (c) The peptide maps generated by partial proteolytic cleavage according to Cleveland (1983) add further, independent evidence for a high degree of similarity between

the soluble and the integral membrane forms of axonin-1. As demonstrated in Fig. 3 D, 26 peptides between 140 and 13 kD resulted from partial proteolytic cleavage with endoproteinase Glu-C, 25 peptides being present in both samples. One peptide of ~22 kD (arrow in Fig. 3 D) was only discernable in the maps derived from membrane-bound axonin-1.

#### ***Axonin-1 Is Distinct from other Adhesive Glycoproteins of the Nervous System***

Axonin-1 was compared with the neuronal cell adhesion molecules of the chicken reported to be associated with neuronal membranes of nerve fiber tracts and involved in axon fasciculation, namely N-CAM, Ng-CAM, G4, F11, and neurofascin (Fig. 4). A comparison with Ng-CAM and N-CAM is shown in the upper three sections of Fig. 4. Both the soluble and the integral membrane form of axonin-1 showed strong reactivity with polyclonal anti-axonin-1 (Fig. 4 A). A strong axonin-1 immunoreactive band was revealed in the vitreous fluid of the eye, whereas the axonin-1-reactive component of the stripped brain membranes was only represented by a relatively faint band. Neither polyclonal anti-Ng-CAM nor polyclonal anti-N-CAM recognized either form of axonin-1 (Fig. 4, B and C, respectively). With polyclonal anti-Ng-CAM (Fig. 4 B, lane BM) and anti-N-CAM antibodies (Fig. 4 C, lane BM) strong immunoreactivity with abundant protein bands of the molecular mass expected for Ng-CAM (Grumet et al., 1984) and N-CAM (Rothbard et al., 1982; Daniloff et al., 1986), respectively, were detected in brain membranes and faint immunoreactivity at the same migratory distance was also observed in the vitreous fluid.

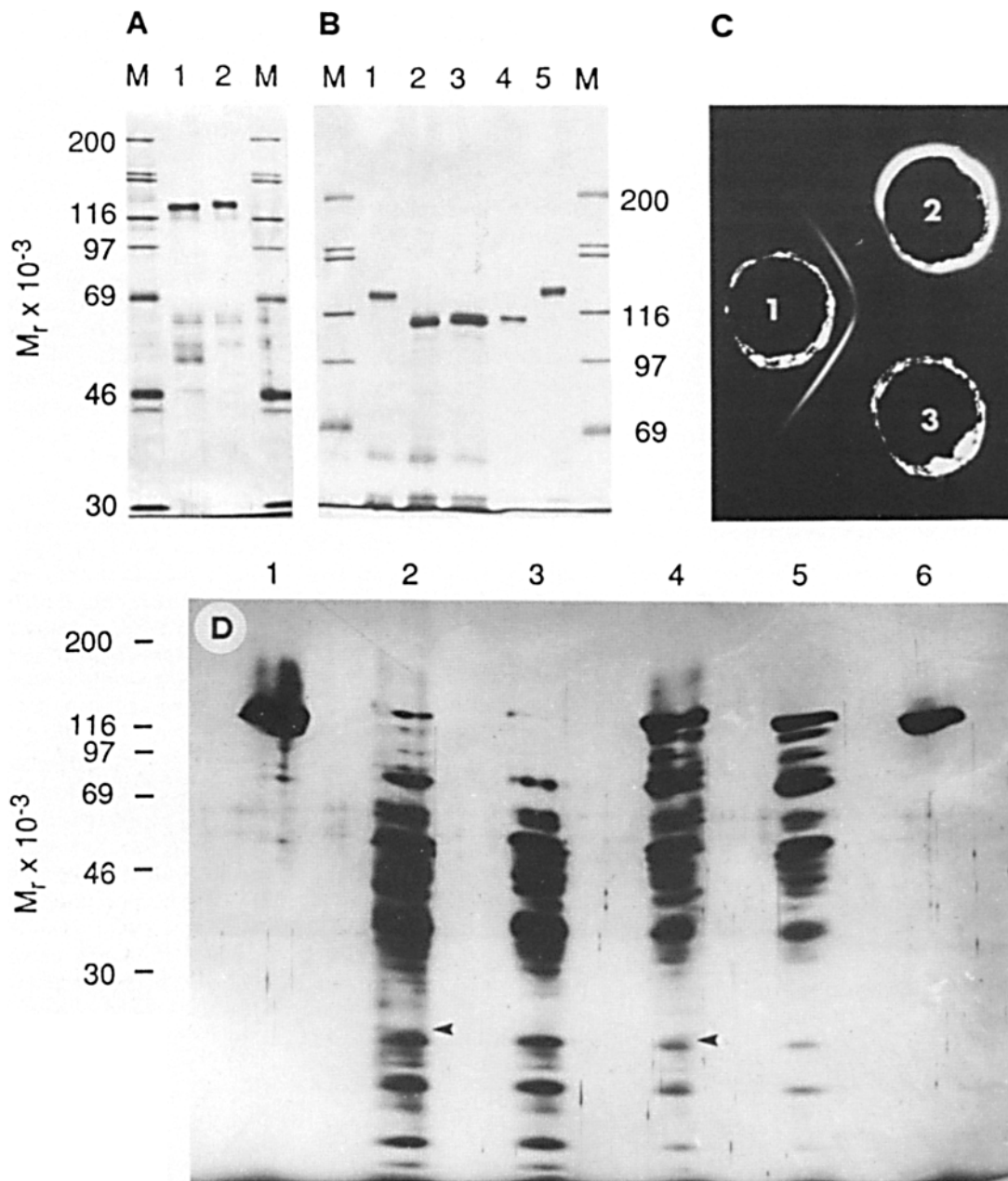
The immunoblot analysis for immunological cross-reactivity of axonin-1 with G4, F11, and neurofascin is shown in the lower three sections of Fig. 4. None of the polyclonal antisera against G4 (D), F11 (E), and neurofascin (F) showed cross-reactivity with soluble axonin-1 or its membrane-bound homologue. Again, as a positive control, the staining pattern of the corresponding immunogen was determined, however, the purified immunogen was used. In all three cases, the obtained band pattern was identical with that reported in the literature (for G4 and F11 cf. Rathjen et al., 1987a; for neurofascin cf. Rathjen et al., 1987b).

#### ***Axonin-1 Has the L2/HNK-1 and the L3 Epitopes in Common with other Adhesive Glycoproteins***

A typification of axonin-1 for adhesion molecules specific carbohydrate structures (Kruse et al., 1984; Kücherer et al., 1987) revealed expression of the L2/HNK-1 as well as the L3 epitope on both the soluble and the membrane-bound forms (see Fig. 5, for an illustration). Two-dimensional SDS-PAGE/immunoblotting further demonstrated the presence of the L2/HNK-1 epitope on all the isoelectric variants (Fig. 5 B).

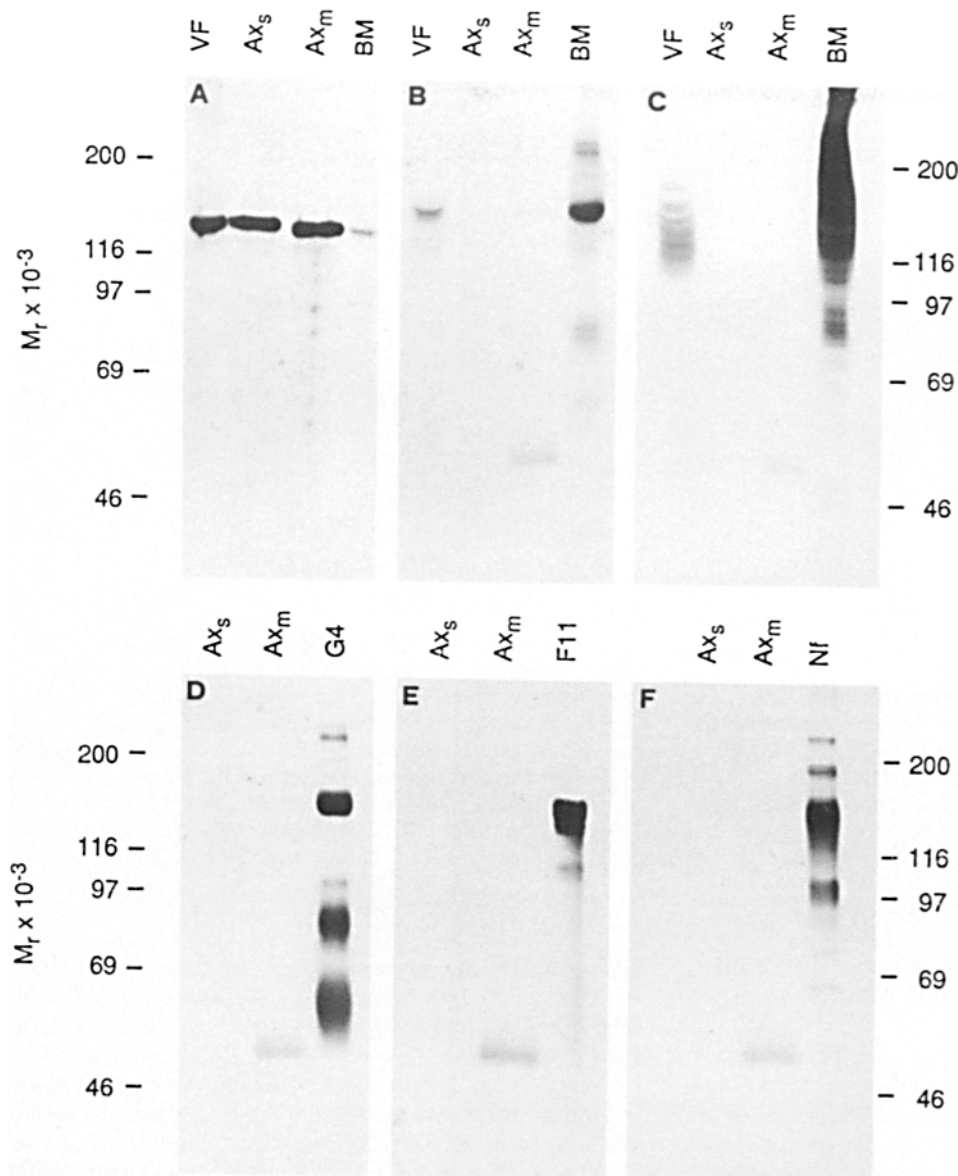
#### ***Pulse-labeling and Pulse-Chase Experiments Reveal Two Cell-associated Forms of Axonin-1, One with Fast and One with Slow Turnover***

As a primary step to address the question whether soluble axonin-1 was derived from the membrane-bound form or independently secreted, the turnover of cellular axonin-1 of cultured neurons was determined by pulse-labeling and pulse-chase experiments (Fig. 6). Preliminary experiments yielded evidence for a rapidly and a slowly saturating form



**Figure 3.** The soluble and the integral membrane forms of axonin-1 are very similar, but not identical. Soluble axonin-1 (Ax<sub>s</sub>) was purified from the vitreous fluid of E14 chicken embryos; the integral membrane form of axonin-1 (Ax<sub>m</sub>) was isolated from stripped brain membranes of 14-d-old chicken embryos by CHAPS solubilization in the presence of protease inhibitors and immunoaffinity chromatography. **A**, a comparison of apparent molecular mass of Ax<sub>s</sub> and Ax<sub>m</sub>. SDS-PAGE, 7.5% with silver staining. Lane 1, 0.25 μg Ax<sub>m</sub>; lane 2, 0.25 μg Ax<sub>s</sub>; lanes M, molecular mass markers. **B**, a comparison of the apparent molecular mass of Ax<sub>s</sub> and Ax<sub>m</sub> after enzymatic removal of N-linked glycans using glycopeptidase F. 5% SDS-PAGE followed by silver staining. Lanes 1 and 5, Ax<sub>m</sub> and Ax<sub>s</sub>, respectively; lanes 2 and 4, N-deglycosylated Ax<sub>m</sub> and Ax<sub>s</sub>, respectively; lane 3, comigration of N-deglycosylated Ax<sub>m</sub> and Ax<sub>s</sub>; lanes M, molecular mass markers. **C**, Ouchterlony double diffusion-in-gel test for immunological comparison of soluble and membrane-bound axonin-1. Well 1 contains 30 μl of rabbit anti-axonin-1 antiserum at a dilution of 1:10. 30 μl of Ax<sub>m</sub> (70 μg/ml) was added to well 2 at the beginning and after 12 and 24 h of incubation. Ax<sub>s</sub> was added to well 3 in three portions as for the integral membrane form. Note the complete fusion of the precipitin lines. Absence of spurs was verified by microscopic inspection using dark field optics with a stereomicroscope (M8, Wild-Leitz). **D**, peptide maps of Ax<sub>s</sub> and Ax<sub>m</sub> according to Cleveland (1983). Lane 1, undigested Ax<sub>m</sub>; lane 2, Ax<sub>m</sub> digested with 0.5% of endoproteinase Glu-C; lane 3, Ax<sub>s</sub> digested with 0.5% of endoproteinase Glu-C; lane 4, Ax<sub>m</sub> digested with 0.1% of endoproteinase Glu-C; lane 5, Ax<sub>s</sub> digested with 0.1% of endoproteinase Glu-C; lane 6, undigested Ax<sub>s</sub>. All peptides generated from Ax<sub>s</sub> are also present in Ax<sub>m</sub>; one additional peptide band occurs only in the cleavage product of Ax<sub>m</sub> (arrow).





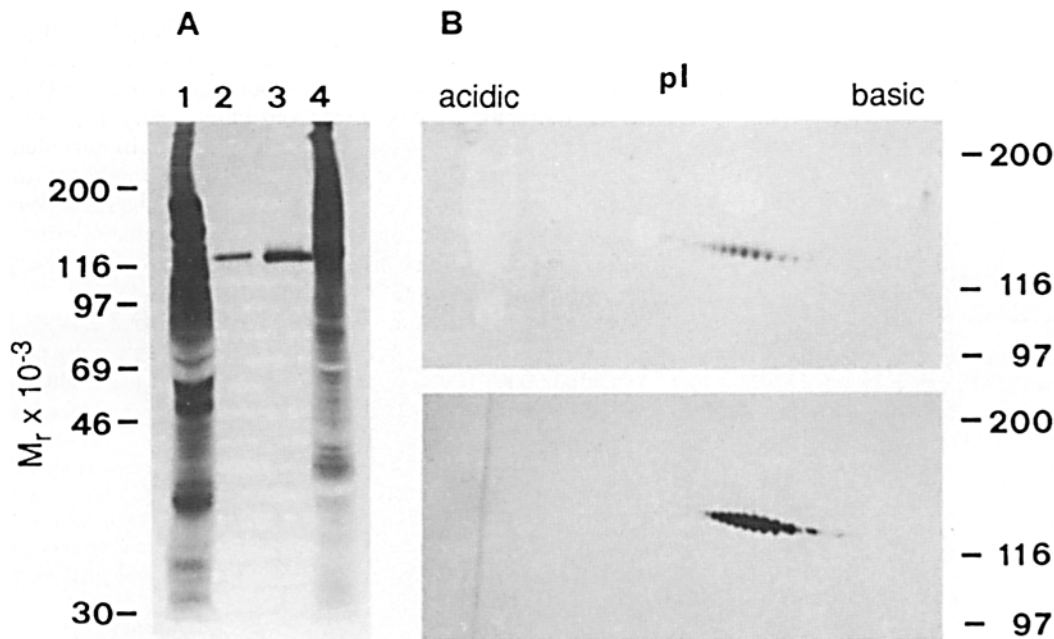
**Figure 4.** Axonin-1 is immunologically distinct from other axon-associated cell adhesion molecules of nerve fiber tracts. (A, B, and C) The cross-reactivity of purified soluble and membrane-bound axonin-1 with polyclonal antibodies against N-CAM and Ng-CAM was investigated by immunoblot analysis; as positive controls, the immunoreactivity of the antibodies with membrane preparations was determined. Identical sets of proteins (lanes 1-4 of each section) were subjected to 7.5% SDS-PAGE followed by electrotransfer onto nitrocellulose. Reactivity with polyclonal antibodies against axonin-1 (A), Ng-CAM (B), and N-CAM (C) was visualized with peroxidase-conjugated second antibodies. Lane VF, 100  $\mu$ g of vitreous fluid proteins; lane  $Ax_s$ , 0.2  $\mu$ g of soluble axonin-1, purified from vitreous fluid of the chicken embryo; lane  $Ax_m$ , 0.2  $\mu$ g of membrane-bound axonin-1, isolated by immunoaffinity chromatography; lane BM, 100  $\mu$ g of CHAPS-extracted brain membrane proteins. Note the prevalence of axonin-1 in the vitreous fluid, where only trace amounts of Ng-CAM and N-CAM are found. Both Ng-CAM and N-CAM are clearly predominant in the brain membranes, where only a minor band of axonin-1 is found. (D, E, and F) The cross-reactivity of purified soluble and membrane-bound axonin-1 with polyclonal antibodies against G4, F11, and neurofascin was investigated by immunoblot analysis. Purified G4, F11,

and neurofascin were used as positive controls. (D) Test for cross-reactivity of soluble and membrane-bound axonin-1 with polyclonal antibodies against G4. Lane  $Ax_s$ , 0.2  $\mu$ g of soluble axonin-1; lane  $Ax_m$ , 0.2  $\mu$ g of membrane-bound axonin-1; lane G4, purified G4 glycoprotein. (E) Test for cross-reactivity of soluble and membrane-bound axonin-1 with polyclonal antibodies against F11. Lane  $Ax_s$ , 0.2  $\mu$ g of soluble axonin-1; lane  $Ax_m$ , 0.2  $\mu$ g of membrane-bound axonin-1; lane F11, purified F11 glycoprotein. (F) Test for cross-reactivity of soluble and membrane-bound axonin-1 with polyclonal antibodies against neurofascin. Lane  $Ax_s$ , 0.2  $\mu$ g of soluble axonin-1; lane  $Ax_m$ , 0.2  $\mu$ g of membrane-bound axonin-1; lane Nf, purified neurofascin.

of cell-associated axonin-1, the slowly saturating form requiring several days to incorporate a reasonable quantity of radioactive amino acid. The turnover rates, thus, were determined by standard decay kinetics after an extended period of labeling. Two populations of axonin-1 were found, as reflected by biphasic kinetics of the decrease of radioactive axonin-1 after the end of the pulse (Fig. 6). Using the data of three independent experiments, half-lives of 5.1 h (SEM = 0.4,  $n = 3$ ) and 104 h (SEM = 11,  $n = 3$ ), respectively, were calculated. Data from decay kinetics being prone to error introduced by isotope reuse (Schimke, 1975), the half-life of the fast-turnover form was also determined by the "approach to equilibrium method", which, for theoretical rea-

sons, is not affected by isotope reuse (Bradley et al., 1976). Only a relatively small discrepancy ( $t_{1/2} = 4.4$  h) was obtained, hence, tracer reuse was concluded to represent a negligible problem in this dissociated cell culture system.

The relative size of the two pools was determined by extrapolation to the beginning of the chase. At this point, when both forms are maximally labeled, the radioactive label is equally distributed between the two kinetic forms. Since, after a pulse of 6 d, the fast-turnover pool is fully labeled, whereas the slow-turnover pool ( $t_{1/2} = 104$  h) is labeled to  $\sim 70\%$ , the estimated size of the slow-turnover pool exceeds that of the fast-turnover pool by a factor close to 1.5.



**Figure 5.** Both membrane-associated and soluble axonin-1 bear the L2/HNK-1 epitope, which has been proposed as a marker for cell adhesion molecules. (A) Immunoblots with monoclonal antibody HNK-1. Lane 1, 100  $\mu$ g of proteins from vitreous fluid; lane 2, 0.2  $\mu$ g of soluble axonin-1, purified from vitreous fluid; lane 3, 0.2  $\mu$ g of membrane-bound axonin-1 isolated by immunoaffinity chromatography from CHAPS-extracts of stripped brain membranes; lane 4, 100  $\mu$ g of CHAPS-extracted stripped brain membrane proteins. (B) Two-dimensional immunoblots with monoclonal antibody HNK-1 and polyclonal anti-axonin-1 antibody. 3  $\mu$ g of soluble axonin-1 were resolved by two-dimensional SDS-PAGE, electrotransferred to nitrocellulose and subsequently stained with HNK-1 (*top*) or anti-axonin-1 antibodies (*bottom*). Note that all the isoelectric variants recognized by anti-axonin-1 also bear the HNK-1 epitope.

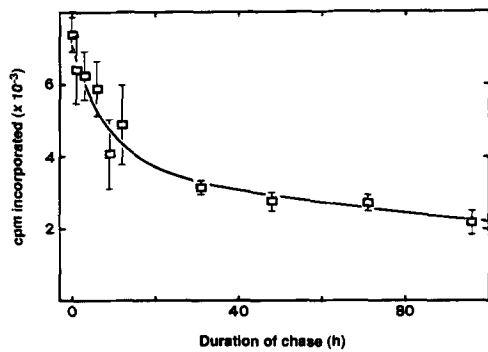
#### **Released Axonin-1 Is Derived from the Fast-Turnover Pool; the Slow-Turnover Pool Does Not Contribute Substantially**

To identify the form representing the pool of origin of released axonin-1, the slow-turnover and the fast-turnover forms were differentially labeled with [ $^3$ H]leucine and [ $^{35}$ S]methionine, respectively, by appropriately timed pulse-chase double-labeling, and the content in each isotope of both the cellular and the released form was determined (Fig. 7). Virtually exclusive labeling of the slow-turnover form was obtained with a long-lasting pulse of [ $^3$ H]leucine (4 d) followed by a chase of 63 h (representing  $>10$  half-lives of the fast-turnover form). Beginning after 63 h of chase, a pulse of [ $^{35}$ S]methionine was given for 48 h. With this pulse duration, full labeling of the fast-turnover pool was obtained; the calculated labeling of the slow-turnover pool was  $\sim 20\%$ .

The radioactivity of [ $^3$ H]leucine and [ $^{35}$ S]methionine found in cellular and released axonin-1, at various points in time during the pulse with [ $^{35}$ S]methionine, is shown in Fig. 7 A. In accordance with the data revealed by the decay kinetics, the cellular form of axonin-1 displayed a biphasic course of isotope incorporation. A fast-saturating phase preceded a period of virtually linear increase, interpreted to represent a slowly saturating second phase. Released axonin-1, after a short lag, had assumed a steady rate of release. An estimation of the relative contributions of each pool to released axonin-1 can be derived from a comparison of the rates of release with the respective pool sizes. The fast-turnover pool of axonin-1 contained, at saturation,  $\sim 2,000$  cpm of [ $^{35}$ S]methionine. Between 11.5 and 48 h of the [ $^{35}$ S]methionine

pulse, the [ $^{35}$ S]methionine-labeled fast-turnover pool had released 4,200 cpm of axonin-1, corresponding to a daily release of 2,800 cpm. Hence,  $\sim 1.4$  times the content of the fast-turnover pool is released per day. In consideration of a half-life of 5.1 h, an estimated 60% of the throughput of the fast-turnover pool was recovered from the extracellular space. The slow-turnover pool of axonin-1 decreased from 3,300 to 2,300 cpm between 63 and 111 h chase. Approximately 500 cpm were recovered with axonin-1 accumulated in the supernatant during this 48-h period. Hence, the slow-turnover pool releases  $<10\%$  of its content in a day. Based on these data, in conjunction with the ratio of the pool sizes determined in the single-isotope pulse-chase studies, the estimated contribution of the fast-turnover pool to axonin-1 release is  $\sim 90\%$ .

The predominant origin of released axonin-1 from the fast-turnover pool is corroborated by the ratios of [ $^{35}$ S]methionine to [ $^3$ H]leucine labeling throughout this prolonged pulse of 48 h in both the cellular and the released axonin-1 (Table II). The ratio in cellular axonin-1 had assumed equivalence by the end of a 48-h pulse period, whereas the ratio in the released form had reached a value  $>10$  at this point. Accounting for the fact that the ratios for the released axonin-1 were derived from cumulative data obtained over the entire pulse period and show a clearly increasing tendency, the instantaneous ratio in axonin-1 released at 48 h was estimated to be considerably higher. Since, under the conditions of this experiment, [ $^3$ H]leucine labeling represents exclusively the slow-turnover pool and most of the [ $^{35}$ S]methionine is located in axonin-1 of the fast-turnover pool, these data clearly



**Figure 6.** Radioactive pulse-chase experiments reveal two kinetically distinct forms of axonin-1. The experimental conditions for these studies were governed to a high degree by the life cycle of the DRG cultures used. Dissociated DRG neurons, when grown in the presence of 5-fluorodeoxyuridine, developed a dense meshwork of axons during the first 6–8 d in culture and thereafter ceased to grow and passed into an apparently stationary period lasting for ~10 d. During this period, neuronal cell death was found not to exceed 20%. Thereafter, however, signs of progressive deterioration became evident. To work under the steady-state assumption, kinetic experiments had to be carried out between day 8 and day 18 in culture. Starting at day 5 in culture, dissociated DRG neurons were pulsed for 6.5 d with 0.25 mCi [<sup>3</sup>H]leucine per milliliter. The medium was removed and cells were washed and chased. At each of the indicated time points of chase, the cells of three cultures were harvested after removal of the medium and axonin-1 was isolated by immunoprecipitation, and analyzed by SDS-PAGE and fluorography. Bands corresponding to axonin-1 were located in the gel by means of fluorography, cut out, and the radioactivity incorporated into axonin-1 was determined by liquid scintillation counting. Each value was determined from triplicate cultures and the vertical bars represent standard deviation.

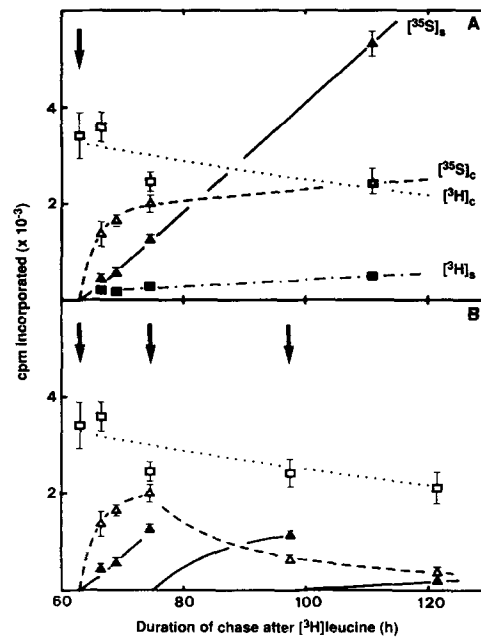
identify the fast-turnover pool as the source of released axonin-1.

The proportion of the fast-turnover pool devoted to release was evidenced during the chase of a short duration (11.5 h) second pulse of [<sup>35</sup>S]methionine (Fig. 7 B). (After a pulse period of 11.5 h, ~6% of the slow-turnover pool ( $t_{1/2} = 104$  h) is labeled, whereas the fast-turnover pool has reached ~75% of saturation.) The release of [<sup>35</sup>S]methionine-labeled axonin-1 during the [<sup>35</sup>S]methionine pulse (63–74.5 h) was 1,243 cpm, i.e., approximately one-half of the estimated pool size of the fast-turnover pool (a lag period of a few hours precedes the period of a linear rate of release). In the two chase periods, the [<sup>35</sup>S]methionine released with axonin-1 was between 70 and 80% of the quantity that had disappeared from the cells: during the first chase of 23 h, 1,360 cpm of [<sup>35</sup>S]methionine had disappeared from the cellular axonin (i.e., the fast-turnover pool) and 1,120 cpm were released; during the second chase of 23 h, 260 cpm disappeared from the cellular axonin-1 and 190 cpm were released. These data are in good agreement with the 60% of the throughput to be attributed to release, as calculated from the data obtained during the 48-h [<sup>35</sup>S]methionine-pulse period of the double-labeling experiment (Fig. 7 A).

#### **The Slow-Turnover Form Is Exposed at the Cell Surface, the Fast-Turnover Form Is Intracellular**

To assess whether there was extracellular exposure of either

one of the kinetically identified forms, externally accessible proteins were covalently labeled by the addition of the amino group-reactive agent biotin-X-NHS to live cells, at time 0 h and time 64 h of chase after a 4-d pulse of [<sup>3</sup>H]leucine (Fig. 8). If biotinylation was carried out immediately at the end of the pulse, ~25% of the labeled axonin-1 was biotinylated. After a chase of 64 h, when the fast-turnover form had virtually disappeared (cf. Fig. 6), ~45% of the remaining slow-turnover form was biotinylated. The absolute amounts of biotinylated axonin-1 at time-points 0 h and 64 h were very similar. The complete disappearance of the fast-turnover form (the white area in the bar representing total axonin-1) between 0 h and 64 h of chase was not accompanied by a corresponding reduction in biotinylated axonin-1; the slight decrease of biotinylated axonin-1 at the later time-point fol-

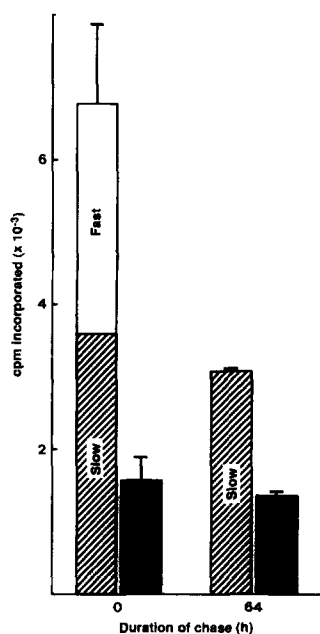


**Figure 7.** Released axonin-1 is derived from the fast-turnover pool; the secretory pathway does not include the slow-turnover pool. (A) After a pulse with 0.25 mCi [<sup>3</sup>H]leucine per milliliter for 4 d and a chase of 63 h, DRG neurons were subjected to a second pulse with [<sup>35</sup>S]methionine (0.25 mCi/ml) for 48 h. (□; [<sup>3</sup>H]<sub>c</sub>) [<sup>35</sup>H]leucine in cellular axonin-1; (■; [<sup>3</sup>H]<sub>s</sub>) [<sup>3</sup>H]leucine in supernatant axonin-1; (Δ; [<sup>35</sup>S]<sub>a</sub>) [<sup>35</sup>S]methionine in cellular axonin-1; and (▲; [<sup>35</sup>S]<sub>s</sub>) [<sup>35</sup>S]methionine in supernatant axonin-1. The arrow at 63 h indicates the beginning of the [<sup>35</sup>S]methionine pulse. (B) Same condition as A, except that the second pulse with [<sup>35</sup>S]methionine was for 11.5 h, followed by two chase periods of 23 h. At the end of the [<sup>35</sup>S]methionine pulse and after each chase period, the culture medium of all cultures was harvested, the cells were washed, and fresh medium was added. The arrow at 63 h indicates the beginning of the [<sup>35</sup>S]methionine pulse; arrows at 74.5 h and 97.5 h indicate the beginning of the consecutive chase periods, respectively. (□) [<sup>3</sup>H]leucine in cellular axonin-1; (Δ) [<sup>35</sup>S]methionine in cellular axonin-1; (▲) [<sup>35</sup>S]methionine in supernatant axonin-1. The values indicated are the mean of triplicate measurements; the vertical bars represent standard deviation. The curve for the released axonin-1 during the first chase period (B) has been arbitrarily drawn for the sake of clarity. The initial slope has been taken from the slope of the release found at this time in the long time pulse of [<sup>35</sup>S]methionine (A).

**Table II. Proportions of the Fast-Turnover and the Slow-Turnover Form in Cellular and Released Axonin-1**

Time	Ratio ([ <sup>35</sup> S]methionine/[ <sup>3</sup> H]leucine)	
	Cellular axonin-1	Supernatant axonin-1
	<i>mean ± SEM</i>	<i>mean ± SEM</i>
<b>Pulse with [<sup>35</sup>S]methionine</b>		
3.5 h	0.38 ± 0.04	2.2 ± 0.1
6 h	0.44 ± 0.02	3.2 ± 0.1
11.5 h	0.81 ± 0.02	4.5 ± 0.2
48 h	1.01 ± 0.07	10.8 ± 0.4
<b>Chase after 11.5-h pulse of [<sup>35</sup>S]methionine</b>		
23 h	0.26 ± 0.02	4.6 ± 0.4
47 h	0.17 ± 0.01	1.1 ± 0.1

DRG neurons were pulsed for 4 d with [<sup>3</sup>H]leucine. After a chase period of 63 h, cells were pulsed with [<sup>35</sup>S]methionine for the time period indicated. The supernatant and cells were harvested separately and axonin-1 was isolated by immunoprecipitation, followed by SDS-PAGE/fluorography. The corresponding bands were cut out of the gels and the ratio of [<sup>35</sup>S]methionine/[<sup>3</sup>H]leucine was determined by liquid scintillation counting.



**Figure 8.** The slow-turnover form of axonin-1 is modified by externally added biotin-X-NHS, i.e., it is membrane surface exposed; the fast-turnover form is protected from external biotinylation, and is therefore presumed to be intracellular. Cultures of dissociated DRG neurons were subjected to an extended pulse with 0.2 mCi [<sup>3</sup>H]leucine per milliliter (4 d), the supernatant was removed and the cells were chased. At the beginning of the chase period and after 64 h, externally accessible proteins of the live cells were modified by reaction with biotin-X-NHS. Biotinylated and nonbiotinylated proteins were separated by chromatography on Streptavidin-agarose. Axonin-1 of both fractions was

immunoprecipitated and subjected to SDS-PAGE/fluorography. The bands corresponding to axonin-1 were located in the gels by means of the fluorographic replica and cut out of the gels. The content of [<sup>3</sup>H]leucine in axonin-1 was determined by liquid scintillation counting. The first bar at each time point represents the total radioactivity of cellular axonin-1. As a visual aid, the proportion of slow-turnover axonin-1 at both time points, as calculated based on pool sizes and turnover rates, is represented by the hatched area of the bar. The solid bar represents the radioactivity of axonin-1 modified by external biotinylation and affinity purified by consecutive subjection to Streptavidin-agarose and immunoprecipitation. All experiments were carried out in triplicate; the vertical lines represent SEM. As a control for the restriction of the labeling to externally exposed proteins, cytosolic aspartate aminotransferase, an enzyme of established cytosolic location (Behra et al., 1982), was included into the study. After exposure of cultured DRG neurons to external biotin-X-NHS, cytosolic aspartate aminotransferase was found to be free of biotin, thus confirming intracellular proteins to be protected from labeling.

lowed the rate of disappearance of the slow-turnover form during this lapse of time. The fact that only ~45% of axonin-1 was biotinylated after 64 h of chase, when only the slow-turnover pool was radioactive, most likely is a matter of yield rather than evidence for two populations of slow-turnover axonin-1. An overall yield of 45% appears reasonable for a procedure comprising a chemical modification of live cells in culture and two steps of affinity chromatographic purification. The aforementioned data clearly demonstrate that at least a substantial proportion of the slow-turnover form is exposed to the cell surface. The fact that the quantity of biotinylated axonin-1 was not higher at time 0 h of chase, when approximately equal quantities of both pools were present, indicates the fast-turnover form to be protected from biotinylation, and hence to be intracellular.

### **Axonin-1 Released from the Fast-Turnover Pool Is Not Exposed on the Cell Surface before Release**

The origin (cell surface vs. intracellular) of released axonin-1 was more directly investigated by external biotinylation of live cells after the second pulse in a pulse-chase double-labeling experiment. As shown in Table III, the released axonin-1 rapidly acquired [<sup>35</sup>S]methionine (the second label) which became prevalent over [<sup>3</sup>H]leucine in <4 h of pulse, as concluded from the ratio of [<sup>35</sup>S]methionine/[<sup>3</sup>H]leucine. The biotinylated, thus externally accessible, cellular axonin-1 showed delayed accumulation of [<sup>35</sup>S]methionine, the isotope ratio remaining strongly in favor of [<sup>3</sup>H]leucine, even after a 10-h pulse. In view of these data, the biotinylated, i.e., the cell surface exposed form of axonin-1, cannot be the source of released axonin-1.

Considering that the measured [<sup>35</sup>S]methionine/[<sup>3</sup>H]leucine ratios of released axonin-1 are derived from cumulative values obtained over the respective times of secretion, and knowing that the released form is derived from the fast-turnover pool, which in this labeling situation contains only [<sup>35</sup>S]methionine, the actual ratios of the cellular precursor pool of released axonin-1 at each time point is expected to be even higher, approaching the value of its precursor pool after a prolonged time, when the initial lag and saturation period becomes negligible. This prediction is fulfilled, if homogeneity of the slow-turnover pool with respect to cell surface exposure is assumed and the data of external biotinylation are corrected for incomplete reaction efficiency. In this case (Table III, last column), the ratio of the released material is indeed below the corresponding ratio of the biotin-free axonin-1 at all time points, reaching a value close to half of the ratio of the intracellular (biotinylation protected) pool after 10 h of pulse, when the labeling of the fast-turnover pool turns towards saturation. These data indicate a precursor-product relationship between the internal, fast-turnover pool and the released axonin-1.

### **Soluble Axonin-1 Is Quantitatively the Major Form**

In embryonic chicken brain membranes, axonin-1 had been found substantially less abundant than Ng-CAM (Fig. 4). Among the soluble proteins of the embryonic vitreous fluid, however, axonin-1 had outranked Ng-CAM by at least one order of magnitude. This observation suggested the major proportion of axonin-1 to be in solution and only a relatively small fraction to occur in membrane-bound form. To obtain

**Table III. External Biotinylation of DRG Neurons during [<sup>35</sup>S]Methionine Pulse of [<sup>3</sup>H]Leucine Pulse-chased Dissociated Cultures**

Duration of [ <sup>35</sup> S]methionine pulse	Form of axonin-1		[ <sup>35</sup> S]Methionine/[ <sup>3</sup> H]leucine	[ <sup>35</sup> S]Methionine/[ <sup>3</sup> H]leucine corrected
<i>h</i>			<i>mean ± SEM</i>	
1	Cellular,	biotin <sup>-</sup>	0.47 ± 0.02	2.1
		biotin <sup>+</sup>	0.02 ± 0.002	0.02
	Supernatant		0	0
4	Cellular,	biotin <sup>-</sup>	1.2 ± 0.1	3.2
		biotin <sup>+</sup>	0.38 ± 0.02	0.38
	Supernatant		1.3 ± 0.1	1.3
10	Cellular,	biotin <sup>-</sup>	1.9 ± 0.3	8.9
		biotin <sup>+</sup>	0.7 ± 0.1	0.7
	Supernatant		4.2 ± 0.3	4.2

DRG neurons were pulsed for 4 d with 0.25 mCi [<sup>3</sup>H]leucine per milliliter. After a chase for 63 h, [<sup>35</sup>S]methionine (0.2 mCi/ml) was added for the time periods indicated. After collecting the supernatant, externally accessible proteins of the live cells were modified by reaction with biotin-X-NHS for 2 min. Subsequently, the cells were washed and lysed. The cellular proteins were subdivided into a biotinylated (i.e., extracellularly exposed) and a nonbiotinylated (i.e., intracellular) fraction by affinity chromatography on Streptavidin-agarose. From both fractions, axonin-1 was isolated by immunoprecipitation, followed by SDS-PAGE/fluorography. Corresponding bands were cut out of the gels, rehydrated, and the [<sup>35</sup>S]methionine/[<sup>3</sup>H]leucine ratio was determined by liquid scintillation counting. In the last column, nonbiotinylated fractions were corrected for the efficiency of this modification-reaction (yield 53%).

a quantitative estimation on the ratio of soluble and membrane-bound axonin-1, the vitreous fluid and the retina of 14-d chicken embryos were investigated for their content in axonin-1. Using ELISA, ~50 ng of axonin-1 were found in the retina, whereas between 1.2 and 2 µg of soluble axonin-1 were present in the vitreous fluid. As a conservative estimate, assuming a constant rate of accumulation between embryonic day 6 and 14 and the absence of degradation, the daily secretion of axonin-1 into the vitreous cavity corresponds to approximately five times the amount of the axonin-1 present in the retinal tissue, the estimated throughput of the secretory pool in the retina being at least three times higher than in the *in vitro* experiments with embryonic DRG neurons. These *in vivo* data confirm the evidence obtained from previous *in vitro* studies (Stoeckli et al., 1989; Ruegg et al., 1989; this paper) that the soluble form of the extracellular space is quantitatively predominant and that only a minor proportion of axonin-1 is found in cell association. Such a quantitative predominance of the soluble over the membrane-associated form in both total quantity and rate of production is unprecedented for cell adhesion molecules reported to date.

## Discussion

### *Membrane-associated Axonin-1, by Operational and Structural Criteria, Is a Cell Adhesion Molecule*

A membrane-associated homologue of the axonally secreted protein axonin-1 has been identified. By its predominant location in nerve fiber tracts, axonin-1 appears closely related to the neural cell adhesion proteins of the L1/nerve growth factor-inducible large external glycoprotein (NILE)/Ng-CAM/G4/8D9-group, F11, and neurofascin, which, because of their common topographic restriction in their expression pattern, recently have been gathered under the denomination AxCAM (Rutishauser and Jessel, 1988). In its occurrence in nerve fiber tracts, axonin-1 overlaps with N-CAM, which however, is found in many other areas of the nervous system

too (Daniloff et al., 1986). Addition of anti-axonin-1 Fab fragments to cultured dorsal root ganglia during the time of axon growth resulted in alterations in the fasciculation pattern, which closely corresponded to those evoked with anti-N-CAM Fab fragments (Rutishauser et al., 1978) and have recently been observed in the presence of antibodies to a number of AxCAMs, namely NILE (Stallcup and Beasley, 1985), L1 (Fischer et al., 1986), Ng-CAM (Friedlander et al., 1986), G4 and F11 (Rathjen et al., 1987a), and neurofascin (Rathjen et al., 1987b). All AxCAMs, as well as N-CAM, have been reported to be membrane proteins, which exert their adhesive function by the binding of their surface-exposed portion to appropriate ligands of adjacent cell membranes. In accordance with this concept, anti-axonin-1 Fab fragments were indeed located at the axonal surface at the end of the fasciculation perturbation experiment, implying axonin-1 contributions to fasciculation in a membrane-associated state. By the facts that it is situated in axonal membranes, exposed to the axonal surface, and its specific antibodies perturb fasciculation, membrane-bound axonin-1 fulfills the operational definition of a cell adhesion molecule (Frazier and Glaser, 1979; Rutishauser and Jessel, 1988).

Besides meeting operational criteria for a cell adhesion molecule, axonin-1 also expresses the L2/HNK-1 and the L3 epitope carbohydrate structures shared by a number of AxCAMs and other Ca<sup>2+</sup>-independent adhesion molecules (Schachner, 1989). The underlying carbohydrate structures have been proposed to represent family traits of adhesive molecules (Kruse et al., 1984; Kücherer et al., 1987), and, recently, evidence for their direct participation as ligands in the adhesive interaction has been presented (Künemund et al., 1988; Schachner, 1989). These structural features, in conjunction with its topographical location and its operational qualification as a cell adhesion molecule, justifies a *bona fide* assignment of axonin-1, in its membrane-bound form, to the AxCAMs.

The nature of the membrane association of axonin-1 was investigated by membrane subfractionation studies. Preparations of embryonic brain membranes were subjected to

procedures designed for the release of vesicle-trapped soluble proteins (ultrasonication), removal of peripheral membrane proteins (high salt and high pH), and the solubilization of integral membrane proteins (CHAPS). A minor proportion of axonin-1 was found in a vesicle-enclosed form, possibly representing the content of secretory vesicles that had copurified with the plasma membranes. The peripheral membrane proteins were solubilized in two fractions by consecutive application of two release procedures of different harshness, to estimate the relative strength of membrane attachment of axonin-1. Exposure to high salt, a relatively mild extraction procedure, was followed by the markedly harsher exposure to high pH (Steck and Yu, 1973; Fujiki et al., 1982). In combination, these procedures removed about one-third (37%) of axonin-1 from the sonicated membranes. The remaining axonin-1 was found releasable by subsequent exposure to CHAPS, and hence operationally qualified as an integral membrane protein. The data on absolute and specific contents of axonin-1 in the brain membrane subfractions reflected a discontinuity in the strength of membrane association. (a) The specific concentration of axonin-1 in the proteins solubilized by the relatively mild high-salt procedure was more than twofold higher than in the material removed by the clearly harsher high pH condition, a fact which indicates this form of axonin-1 to be among the peripheral membrane proteins with a relatively weak membrane attachment. (b) Among the proteins resistant to high pH release and only extractable by CHAPS, the specific concentration of axonin-1 was >12-fold higher than among the high pH-released proteins, indicating this form to be among the most tightly bound membrane proteins. Therefore, with respect to the relative strength of membrane attachment, a very weakly bound peripheral membrane form of axonin-1 is clearly distinct from an integral membrane form with a very strong membrane association.

The integral membrane form of axonin-1 was compared with the soluble form by physicochemical, immunological, and protein-chemical criteria. The fact that the soluble and the integral membrane forms of axonin-1 are identical with respect to their isoelectric variant patterns, show complete fusion of their precipitin lines in the Ouchterlony double-diffusion test, and generate highly similar peptide patterns upon partial proteolytic cleavage, indicate a very high degree of structural similarity. However, a distinct difference in the relative migratory distance was observed on SDS-PAGE. The membrane-associated form characterized as an integral membrane protein displayed a lower apparent molecular mass than its soluble counterpart. The identity of the antigenic determinants and the identity of all but one out of 26 proteolytic peptides, suggests that the difference in the electrophoretic migration rate may result from a localized structural difference of two otherwise identical proteins. A difference in the length of the polypeptide chain at either end of the molecule is a conceivable explanation but also a local difference arising from posttranslational modification may be considered. The molecular mass difference was maintained after enzymatic removal of N-linked glycans, indicating the underlying structural difference not to reside in N-linked carbohydrate. The qualification of a protein as an integral membrane protein based on resistance to solubilization by high salt or high pH is operational and hence, no conclusions on the nature of the membrane attachment are possi-

ble. The apparent molecular mass of the membrane-bound form being smaller than that of soluble axonin-1, membrane anchorage by covalently attached lipid is conceivable (Ferguson and Williams, 1988).

***The Soluble Form of Axonin-1 Is Not Derived from the Membrane Form, but Directly Secreted from an Intracellular Pool; As a Soluble Molecule Interacting with Adhesion Receptors, It Might Modulate Adhesive Interactions***

Cell adhesion molecules, by definition, are considered to exert their function in membrane-association, however, a possible role in solution has been suggested by the observation of soluble forms of N-CAM (Thiery et al., 1977; Goridis et al., 1978; Rohrer and Schachner, 1980) and NILE (Salton et al., 1983; Stallcup et al., 1983; Sweadner, 1983) in the conditioned media of neuronal cultures. The release of NILE (which is the rat homologue to LI, Ng-CAM, G4, and 8D9; Rutishauser and Jessel, 1988) and of another cell surface glycoprotein, possibly corresponding to N-CAM, was found to be evoked or enhanced by conditions which favor transmitter secretion and the mechanism of release was investigated (Sweadner, 1983). Shedding from the cell surface rather than classical secretion was found to underly this protein release, as evidenced by the fact that the released form was found to be derived from a higher molecular weight, membrane-bound, and surface exposed precursor (Sweadner, 1983). Soluble axonin-1, in contrast, is not exposed to the cell surface before its release, but directly originates from an intracellular pool. This observation speaks in favor of classical secretion after packaging into secretory vesicles as the mechanism of release.

Dualistic expression of a protein in a secreted and a membrane-bound form has previously been observed, e.g., with immunoglobulins (Cushley et al., 1982) and muscle acetylcholinesterase (Rotundo and Fambrough, 1980). Secreted acetylcholinesterase, in accordance with our present findings on secreted axonin-1, was also derived from a rapidly turning-over, intracellular pool, and, as membrane-bound axonin-1, the membrane-inserted form of the esterase had a markedly longer half-life (Rotundo and Fambrough, 1980). Whether both secreted and membrane-bound axonin-1 are expressed by the same cell at the same time, and whether there is independent regulation of their expression, remains to be determined.

Secreted axonin-1 occurs in relatively high concentrations in embryonic cerebrospinal fluid and vitreous fluid (Ruegg et al., 1989) and, with respect to the overall abundance in the central nervous system, is clearly the prevalent form. This is in notable contrast to the distribution of the other cell adhesion molecules that were investigated in this respect, namely N-CAM or Ng-CAM, where the major proportion is membrane associated and only trace amounts can be detected in solution (Fig. 4, A-C). Although a functional role of secreted axonin-1 has not been determined thus far, the molecule carries features that suggest it to have adhesive properties. (a) Secreted axonin-1 is structurally very similar to its membrane-bound homologue, which is involved in neurite fasciculation. (b) The antibodies evoking the observed alterations in the fasciculation pattern, have been raised against the soluble antigen. Hence, the structure un-

derlying the antibody perturbation effect seems to be expressed on both forms. (c) As its membrane-bound homologue, secreted axonin-1 carries both the L2/HNK-1 and the L3 epitopes.

The possibility of cell adhesive properties on a soluble glycoprotein secreted from axons during neurogenesis encourages speculation about its functional role. Depending on its molecular configuration, a soluble adhesion molecule may have inverse functions. By binding in a soluble monovalent form to a cell surface or ECM receptor, it could effect inhibition of cell adhesion, whereas in a bivalent form, it could act as a cross-linker between membranes or between membranes and the ECM, hence promoting cell adhesion. In the light of these suppositions, the membrane surface-adsorbed form of axonin-1 (released by high salt and high pH) could be interpreted to represent receptor-bound soluble axonin-1. The adventitious trapping of soluble axonin-1 from the extracellular fluid during the membrane preparation process cannot be excluded on the basis of the currently available data, and therefore, unequivocal identification and localization of *in vivo* membrane surface-bound secreted axonin-1 by immunohistochemical staining in tissue sections is necessary. Yet, as a working hypothesis, it seems reasonable to assume that the intense staining along nerve fiber tracts reflects the integral membrane form, because this form represents the quantitatively major form of axonin-1 found in membranes. Since virtually all reported observations locate the site of axonal protein secretion to the axon tip (Kelly, 1988), the peripherally adsorbed form of membrane-associated axonin-1 may occur in areas of high growth cone densities. Appropriate binding sites provided, high local concentrations in the narrow extracellular space in proximity of the site of release would favor preferential binding to cell surfaces and/or ECM. Staining of the inner plexiform layer of the retina (Fig. 1 C) with its high density of growth cones at this stage of development represents an intriguing observation in this context, as it could reflect secreted axonin-1. Both ultrastructural localization of the site of axonin-1 secretion from growth cones of elongating axons and its extracellular location will be necessary to test this hypothesis. Further studies will also include investigations on the ligand function of axonin-1 and its role in neural development. Preparative efforts to produce sufficient quantities of pure axonin-1 for studies towards this goal, are currently in progress in our laboratory.

We are indebted to Dr. Fritz Rathjen for providing G4, F11, neurofascin, and their respective antibodies; Drs. Gerald Edelman, Kathryn L. Crossin, and Martin Grumet for antibodies against N-CAM and Ng-CAM; and Dr. Melitta Schachner and collaborators for monoclonal antibodies against the L2 and the L3 epitopes. We thank Dr. Heinz Gehring for providing cytosolic aspartate aminotransferase, the corresponding antiserum and the many helpful suggestions; Marika Born-Kaufmann for the excellent technical assistance; and Colin J. Henehan for reading the manuscript.

We acknowledge the financial support of the work presented here by the Swiss National Science Foundation, the Geigy-Jubiläumstiftung, the EMDO-Stiftung, the Hartmann Müller-Stiftung, the Sandoz-Stiftung, the Schweizerische Multiple Sklerose Gesellschaft, and the Jubiläumstiftung der Schweizerischen Lebensversicherungs- und Rentenanstalt für Volksgesundheit und medizinische Forschung.

Received for publication 3 May 1989 and in revised form 26 June 1989.

## References

- Behra, R., P. Christen, M. A. Spycher, and P. Sonderegger. 1982. Subcellular distribution of aspartate aminotransferase isoenzymes in chicken heart: a quantitative study. *Comp. Biochem. Physiol.* 71B:187-191.
- Böhlen, P., S. Stein, W. Dairman, and S. Udenfriend. 1973. Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* 155:213-220.
- Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
- Boxberg, von, Y. 1987. Untersuchungen über Membranoberflächen- und Extrazellulärmatrixproteine im Hinblick auf ihre Beteiligung an der Embryogenese der retino-tectalen Projektion beim Hühnchen. Ph.D. thesis. University of Tübingen, Tübingen, FRG. 86 pp.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Bradley, M. O., L. Hayflick, R. T. Schimke. 1976. Protein degradation in human fibroblasts (WI-38). *J. Biol. Chem.* 254:3521-3529.
- Burden, S. J., P. B. Sargent, and U. J. McMahan. 1979. Acetylcholine receptors in regenerating muscle accumulate at original synaptic sites in the absence of the nerve. *J. Cell Biol.* 82:412-425.
- Campanot, R. B. 1977. Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 74:4516-4519.
- Carlson, S. S., P. Caroni, and R. B. Kelly. 1986. A nerve terminal anchorage protein from electric organ. *J. Cell Biol.* 103:509-520.
- Caroni, P., S. S. Carlson, E. Schweitzer, and R. B. Kelly. 1985. Presynaptic neurones may contribute a unique glycoprotein to the extracellular matrix at the synapse. *Nature (Lond.)* 314:441-443.
- Cleveland, D. W. 1983. Peptide mapping in one dimension by limited proteolysis of sodium dodecyl sulfate-solubilized proteins. *Methods Enzymol.* 96:222-229.
- Cushley, W., B. E. H. Coupar, C. A. Mickelson, and A. R. Williamson. 1982. A common mechanism for the synthesis of membrane and secreted immunoglobulin alpha, gamma and mu chains. *Nature (Lond.)* 298:77-79.
- Daniloff, J. K., C.-M. Chuong, G. Levi, and G. M. Edelman. 1986. Differential distribution of cell adhesion molecules during histogenesis of the chick nervous system. *J. Neurosci.* 6:739-758.
- Ferguson, M. A. J., and A. F. Williams. 1988. Cell-surface anchoring of proteins via glycosylphosphatidylinositol structures. *Annu. Rev. Biochem.* 57:285-320.
- Fischer, G., V. Künemund, and M. Schachner. 1986. Neurite outgrowth patterns in cerebellar microexplant cultures are affected by antibodies to the cell surface glycoprotein L1. *J. Neurosci.* 6:605-612.
- Frazier, W., and L. Glaser. 1979. Surface components and cell recognition. *Annu. Rev. Biochem.* 48:491-523.
- Friedlander, D. R., M. Grumet, and G. M. Edelman. 1986. Nerve growth factor enhances expression of neuron-glia cell adhesion molecule in PC12 cells. *J. Cell Biol.* 102:413-419.
- Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97-102.
- Godfrey, E. W., R. M. Nitkin, B. G. Wallace, L. L. Rubin, and U. J. McMahan. 1984. Components of *Torpedo* electric organ and muscle that cause aggregation of acetylcholine receptors on cultured muscle cells. *J. Cell Biol.* 99:615-627.
- Goridis, C., M. A. Joher, M. Hirsch, and M. Schachner. 1978. Cell surface proteins of cultured brain cells and their recognition by anti-cerebellum (anti-NS-4) antiserum. *J. Neurochem.* 31:531-539.
- Grumet, M., S. Hoffman, C.-M. Chuong, and G. M. Edelman. 1984. Polypeptide components and binding functions of neuron-glia cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 81:7989-7993.
- Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49-92.
- Hare, J. F., and E. Lee. 1989. Metabolic behavior of cell surface biotinylated proteins. *Biochemistry.* 28:574-580.
- Hawkes, R., E. Niday, and J. Gordon. 1982. A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* 119:142-147.
- Hoffman, S., B. C. Sorkin, P. C. White, R. Brackenbury, R. Mailhammer, U. Rutishauser, B. C. Cunningham, and G. M. Edelman. 1982. Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J. Biol. Chem.* 257:7720-7729.
- Kelly, R. B. 1988. The cell biology of the nerve terminal. *Neuron.* 1:431-438.
- Kreibich, G., and D. D. Sabatini. 1974. Selective release of content from microsomal vesicles without membrane disassembly. II. Electrophoretic and immunological characterization of microsomal subfractions. *J. Cell Biol.* 61:789-807.
- Kruse, J., R. Mailhammer, H. Wernecke, A. Faissner, I. Sommer, C. Goridis, and M. Schachner. 1984. Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature (Lond.)* 311:153-155.
- Krystosek, A., and N. W. Seeds. 1981. Plasminogen activator release at the neuronal growth cone. *Science (Wash. DC)* 213:1532-1534.
- Krystosek, A., and N. W. Seeds. 1984. Peripheral neurons and schwann cells

- secrete plasminogen activator. *J. Cell Biol.* 98:773-776.
- Kücherer, A., A. Faissner, and M. Schachner. 1987. The novel carbohydrate epitope L3 is shared by neural cell adhesion molecules. *J. Cell Biol.* 104:1597-1602.
- Künemund, V., F. B. Jungalwala, G. Fischer, D. K. Chou, G. Keilhauer, and M. Schachner. 1988. The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. *J. Cell Biol.* 106:213-223.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lemmon, V., and S. C. McLoon. 1986. The appearance of an L1-like molecule in the chick primary visual pathway. *J. Neurosci.* 6:2987-2994.
- Magill-Solc, C., and U. J. McMahan. 1988. Motor neurons contain agrin-like molecules. *J. Cell Biol.* 107:1825-1833.
- Nilsson, L.-A. 1983. Double diffusion-in-gel. *Scand. J. Immunol.* 10(suppl.): 57-68.
- Nitkin, R. M., M. A. Smith, C. Magill, J. R. Fallon, Y. M. M. Yao, B. G. Wallace, and U. J. McMahan. 1987. Identification of agrin, a synaptic organizing protein from *Torpedo* electric organ. *J. Cell Biol.* 105:2471-2478.
- Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361-363.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- Pittman, R. N. 1985. Release of plasminogen activator and a calcium-dependent metalloprotease from cultured sympathetic and sensory neurons. *Dev. Biol.* 110:91-101.
- Porter, R. R. 1959. The hydrolysis of rabbit  $\gamma$ -globulin and antibodies with crystalline papain. *Biochem. J.* 73:119-126.
- Rathjen, F. G., J. M. Wolff, R. Frank, F. Bonhoeffer, and U. Rutishauser. 1987a. Membrane glycoproteins involved in neurite fasciculation. *J. Cell Biol.* 104:343-353.
- Rathjen, F. G., J. M. Wolff, S. Chang, F. Bonhoeffer, and J. A. Raper. 1987b. Neurofascin: a novel chick cell-surface glycoprotein involved in neurite-neurite interactions. *Cell.* 51:841-849.
- Rohrer, H., and M. Schachner. 1980. Surface proteins of cultured mouse cerebellar cells. *J. Neurochem.* 35:792-803.
- Rothbard, J. B., R. Brackenbury, B. A. Cunningham, and G. M. Edelman. 1982. Differences in the carbohydrate structures of neural cell-adhesion molecules from adult and embryonic chicken brains. *J. Biol. Chem.* 257:11064-11069.
- Rotundo, R. L., and D. M. Fambrough. 1980. Synthesis, transport, and fate of acetylcholine esterase in cultured chick embryo muscle cells. *Cell.* 22:583-594.
- Ruegg, M. A., E. T. Stoeckli, T. B. Kuhn, M. Heller, R. Zuellig, and P. Sonderegger. 1989. Purification of axonin-1, a protein that is secreted from axons during neurogenesis. *EMBO (Eur. Mol. Biol. Org.) J.* 8:55-63.
- Rubin, R. W., and R. W. Warren. 1977. Quantitation of microgram amounts of proteins in SDS-mercaptoethanol-Tris electrophoresis sample buffer. *Anal. Biochem.* 83:773-777.
- Rutishauser, U., W. E. Gall, and G. M. Edelman. 1978. Adhesion among neural cells of the chick embryo. IV. Role of the cell surface molecule CAM in the formation of neurite bundles in cultures of spinal ganglia. *J. Cell Biol.* 79:382-393.
- Rutishauser, U., and T. M. Jessel. 1988. Cell adhesion molecules in vertebrate neural development. *Physiol. Rev.* 68:819-857.
- Salton, S. R. J., C. Richter-Landsberg, L. A. Greene, and M. L. Shelanski. 1983. Nerve growth factor-inducible large external (NILE) glycoprotein: studies of a central and peripheral neuronal marker. *J. Neurosci.* 3:441-454.
- Schachner, M. 1989. Families of neural adhesion molecules. *J. Cell. Biochem.* (Suppl. 13, Part. A.) In press.
- Schimke, R. T. 1975. Methods for analysis of enzyme synthesis and degradation in animal tissues. *Methods Enzymol.* 40:241-266.
- Snow, P. M., K. Zinn, A. H. Harrelson, L. McAllister, J. Schilling, M. J. Bastiani, G. Makk, and C. S. Goodman. 1988. Characterization and cloning of fasciclin I and fasciclin II glycoproteins in the grasshopper. *Proc. Natl. Acad. Sci. USA.* 85:5291-5295.
- Sonderegger, P., M. C. Fishman, M. Bokoum, H. C. Bauer, E. A. Neale, and P. G. Nelson. 1984. A few axonal proteins distinguish ventral spinal cord neurons from dorsal root ganglion neurons. *J. Cell Biol.* 98:364-368.
- Sonderegger, P., P. F. Lemkin, L. E. Lipkin, and P. G. Nelson. 1985. Differential modulation of the expression of axonal proteins by non-neuronal cells of the peripheral nervous system. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1395-1401.
- Stallcup, W. B., and L. Beasley. 1985. Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain. *Proc. Natl. Acad. Sci. USA.* 82:1276-1280.
- Stallcup, W. B., L. S. Arner, and J. M. Levine. 1983. An antiserum against PC12 cell line defines cell surface antigens specific for neurons and Schwann cells. *J. Neurosci.* 3:53-68.
- Steck, T. L., and J. Yu. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. *J. Supramol. Struct.* 1:220-232.
- Stoeckli, E. T., P. F. Lemkin, T. B. Kuhn, M. A. Ruegg, M. Heller, and P. Sonderegger. 1989. Identification of proteins secreted from axons of embryonic dorsal root ganglia neurons. *Eur. J. Biochem.* 180:249-258.
- Sweadner, K. J. 1983. Post-translational modification and evoked release of two large surface proteins of sympathetic neurons. *J. Neurosci.* 3:2504-2517.
- Switzer, R. C., III, C. R. Merrill, and S. Shifrin. 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem.* 98:231-237.
- Thiery, J.-P., R. Brackenbury, U. Rutishauser, and G. M. Edelman. 1977. Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. *J. Biol. Chem.* 252:6841-6845.
- Thiery, J.-P., A. Delouvé, M. Grumet, and G. M. Edelman. 1985. Initial appearance and regional distribution of the neuron-glia cell adhesion molecule in the chick embryo. *J. Cell Biol.* 100:442-456.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Wessel, D., and U. I. Flügge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138:141-143.