Neurofilament-Like Immunoreactivity in the Sea Anemone *Condylactis gigantea* (Cnidaria: Anthozoa)

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Abstract. The neuronal cytoskeleton contains neurofilament proteins that serve as markers for nervous tissue in many species across phyla. Antiserum generated to mammalian neuronlaments was used for immunocytochemical staining of tissues in the sea anemone *Condylactis gigantea* (Cnidaria: Anthozoa). Specific staining, visible at the light and electron microscope levels, was found in the tissues of the tentacle. Proteins were extracted from the tissues and solubilized. SDS-polyacrylamide gel electrophoresis and Western blotting revealed two bands of MW r 156 kD and 74 kD that reacted with antiserum generated to neuronlaments. The protein bands also bound a monoclonal antibody shown to react with a highly conserved epitope in many classes of intermediate filaments. These data suggest that the neurons of this anthozoan contain neurofilament-like proteins with molecular properties similar to those of mammalian neurons.

Introduction

Nervous systems probably evolved in animals related to members of present-day cnidarians. The nervous system in these primitive metazoans has been described as consisting of diffuse nets of sensory, motor, and neurosecretory cells that provide connections within the epithelial layers (Bullock and Horridge, 1965).

Neuronal cells and processes contain a filamentous framework that forms the cytoskeleton. Neurofilaments (NFs), a class of structures that express a head-rod-tail organization, belong to the family of intermediate filaments (IFs) and are major components of the neuronal cytoskeleton (Wang et al., 1985; Steinert and Roop, 1988). The central helical rod domain has a hydrophobic backbone that causes aggregation of the subunits into coiled-coil 10-nm filamentous structures; this domain also contains an evolutionarily conserved region, a major feature found in identified IF proteins (Pruss et al., 1981; Geisler and Weber, 1982; Hanukoglu and Fuchs, 1983; Steinert et al., 1983). Besides playing a central role in the structural matrix of neurons, NFs are of particular interest because they may be determining factors in the development of the central nervous system.

The presence and location of these filaments have been visualized by various methods, including histological staining and immunocytochemistry (Phillips et al., 1983; Lasek et al., 1985). NFs have been isolated from many cell types (Cooke and Chase, 1971; Daniels and Huneeus, 1973; Brown et al., 1976) and a variety of species (Lasek et al., 1979; Moon et al., 1981; Phillips et al., 1983). Mammalian neurofilaments are composed of three polypeptides (the “triplet” proteins), with molecular weights of approximately 200 kD (NF-H), 160 kD (NF-M), and 70 kD (NF-L) (Kaufmann et al., 1984; Scott et al., 1985). Some non-mammalian species have triplet proteins that are homologous to those found in mammals. Others, however, have only a single high molecular weight subunit that is immunologically similar to both NF-H and NF-M (Shaw et al., 1984; Shaw, 1991). In invertebrates, neurofilaments are even more variable than their vertebrate counterparts (Eagles et al., 1981; Shaw, 1991; Szaro et al., 1991; Way et al., 1992).

The cytoskeletal components that form an early metazoan nervous system, such as the one found in members of the phylum Cnidaria, are not well-defined. We use im-

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munocytochemical techniques, as well as protein isolation, light microscopy, and transmission electron microscopy (TEM), in our studies of the nervous system in these lower organisms. In this investigation, we focused on confirming the presence of neurofilament-like antigens in the tissues of the subtropical sea anemone *Condylactis gigantea* (Cnidaria: Anthozoa). Our studies suggest that neurons of this anthozoan contain neurofilament-like proteins with molecular properties similar to those of higher species.

**Materials and Methods**

**Animals**

Specimens of *Condylactis gigantea* were obtained from a supplier in Los Angeles, California, and kept in aerated, filtered, recirculating aquaria at 24°C in natural seawater from the Catalina Marine Science Center (Santa Catalina Island, California). Animals acquired from local suppliers in Philadelphia, Pennsylvania, were kept in aerated, filtered artificial seawater. All animals were maintained on a 12/12 photoperiod and fed *Artemia* nauplii, small pieces of squid, or both three times a week. Water was changed at two-week intervals.

Specimens were relaxed by immersion in seawater containing succinylcholine chloride (67 μg/ml, Sigma Chemical Co., St. Louis, Missouri). The tentacles of these relaxed animals were of about normal length, but the diameter was somewhat smaller than normal. They were clamped with a hemostat at the junction of the oral disk and excised.

**Bodian silver stain**

The Bodian silver stain method allows visualization of neural tissue in many species (Bodian, 1936; Gambetti et al., 1981). Bodian silver stain was applied to tissue sections as described by Clark (1981) and to SDS polyacrylamide gels after electrophoresis of proteins as described by Gambetti et al. (1981).

**Light microscopic immunocytochemistry**

Excised tentacles were placed in 2% paraformaldehyde in phosphate buffered saline (PBS; 0.1 M sodium phosphate buffer, 0.9% NaCl), pH 6.5, for 2 h at room temperature and were postfixed in 2% paraformaldehyde in PBS, pH 11 (Berod et al., 1981), for 8 h at 4°C. Samples were rinsed three times in PBS, pH 7.4, and cryoprotected by immersion for 12 h each in 10, 20, and 30% sucrose in PBS, pH 7.4, at 4°C. Tissue was placed in Tissue-Tek (Miles Inc., Elkhart, Indiana) and frozen in isopentane at liquid nitrogen temperatures. Twenty micrometer sections were made on a Microm 500 M Cryostat (Zeiss Inc., Thornwood, New York) at −20°C and thaw-mounted on slides.

Slides were placed in PBS for 20 min and endogenous peroxidases were inactivated with 0.3% hydrogen peroxide in PBS for 20 min. After being rinsed in three successive 15-min changes of PBS, the specimens were incubated for 1 h in PBS containing 0.3% Triton X-100 and 10% normal serum (Vector Laboratories, Burlingame, California), then placed in primary antiserum [polyclonal neurofilament 200 (NF 200, Chemicon International, Temecula, California, and Sigma Chemical Co., St. Louis, Missouri)] at a 1:20,000 dilution for 12 h at 4°C. Three 15-min rinses in PBS were followed by incubation with a biotinylated secondary antibody, several PBS rinses, and incubation with a preformed avidin/biotinylated horse-radish peroxidase complex (Vector Laboratories, Burlingame, California) following the manufacturer’s instructions. Tissues were rinsed two successive times for 10-min each in freshly made acetate/imidazole buffer (0.01 M imidazole, 0.05 M sodium acetate, pH 7.4) and transferred to a nickel chromagen [0.095 M nickel (II) sulfate, 0.05% DAB, 0.03% hydrogen peroxide] in acetate/imidazole buffer to develop.

Control procedures included deletion of secondary antibodies and replacement of the primary antiserum with normal serum.

**Electron microscopic immunocytochemistry**

Portions of tentacles were placed in 4.0% paraformaldehyde/0.5% glutaraldehyde in 0.1 M PBS, pH 6.5, for 1 h at room temperature and transferred to 4.0% paraformaldehyde/0.5% glutaraldehyde, pH 11, for 8 h at 4°C before washing in PBS, pH 7.4, and dehydration in a graded ethanol series. Samples were infiltrated and embedded in EM-BED-812 (Electron Microscopy Sciences, Ft. Washington, Pennsylvania) according to manufacturer’s directions. Silvers to gold sections were placed on nickel grids (Ted Pella Inc., Redding, California). Grids were floated on small amounts of reagent at room temperature as follows: (1) 1 h on 0.1 M PBS at pH 7.4; (2) 5 min on a saturated solution of sodium metaperiodate, followed by 1 h on several changes of PBS; (3) 1 h on 10% normal serum (Vector Laboratories, Burlingame, California); (4) three successive 30-min rinses on PBS; and (5) 2 h on antiserum to polyclonal NF 200 (Sigma Chemical Co., St. Louis, Missouri). Grids were rinsed on three successive changes of PBS and floated on a 1:40 dilution of gold-conjugated IgG (Janssen Life Sciences Products, Piscataway, New Jersey) for 1 h, followed by two successive 20-min distilled water rinses. Grids were postfixed for 15 min on a drop of 2% glutaraldehyde and poststained in lead citrate and uranyl acetate, followed by two successive 15-min rinses on distilled water. Specimens were
examined and photographed in a JEOL 100CXII electron microscope (JEOL USA Inc., Peabody, Massachusetts).

Controls consisted of replacement of the primary antisem with normal serum.

Isolation of neurofilament-like proteins

To demonstrate the existence of neurofilament-like components in the sea anemone *C. gigantea*, we isolated the constituent proteins. To minimize protein contamination, food was withheld from anemones for three days before extraction. Whole anemones were cleaned of debris and weighed. Specimens were chilled for 15 min at −70°C before being quickly chopped into 1-cm³ pieces and placed in SEDTA (20 mM Na-ACES buffer, pH 7.4, 0.3 M sucrose, 2 mM EDTA) for 15 min with intermittent gentle hand agitation to remove mucus. Minced tissue was transferred to TME medium [10 mM tris(hydroxy-methyl)-aminomethane (Tris) HCl, 3 mM MgCl₂, 2 mM K₂-ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N' -tetraacetic acid (EGTA), pH 8.2] and homogenized, with the aid of a Teflon-glass homogenizer, in the presence of 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM io-dodecyl sulfate (SDS). Pelleted material was resuspended in TME containing 2% SDS, homogenized in a Teflon-glass homogenizer, and centrifuged at 9000 × g for 2 h at 0°C and the supernatant (S1) reserved. The pellet was successively washed four times in ice cold TME with a brief centrifugation after each wash at 9000 × g for 15 min at 0°C. Because of the difficulty encountered in solubilizing cytoskeletal components in Triton X-100 in early experiments, all subsequent procedures utilized sodium dodecyl sulfate (SDS). Pelleted material was resuspended in TME containing 2% SDS, homogenized in a Teflon-glass homogenizer, and centrifuged at 9000 × g for 2 h at 0°C. The supernatant (S2) was removed and stored at −70°C. The pellet (P2) was washed in TME and re-extracted in SDS. Any remaining insoluble material was removed by centrifugation at 9000 × g for 2 h at 0°C, and the supernatant (S3) stored at −70°C. Protein content was determined by the method of Bradford (1976).

Control procedures consisted of extraction of rat brain as described above.

Absorption of the neurofilament-like extract

In several experiments, polyclonal NF 200 antibodies were absorbed with the washed resuspension of neurofilament-like protein (S2, above) from which the SDS had been removed (Suzuki and Terada, 1988) prior to use. The preparation, consisting of the extract of neurofilament-like proteins and the added antibodies, was placed at 4°C for 24 h with gentle stirring. The supernatant was decanted after centrifugation at 9500 × g for 2 h at 0°C and was used for immunostaining.

**SDS-polyacrylamide gel electrophoresis and Western blotting**

Proteins were electrophoresed in the presence of SDS on 12.5% and 4/20% gradient gels by the method of Laemmlı (1970) and transferred to nitrocellulose sheets. Western blotting was performed by the method of Towbin et al. (1979). In brief, blots were processed through incubations with blocking solution (5% skim milk in 0.1 M PBS/0.1% Tween-20) for 12 h at 4°C, and immunostained with one of the following: (1) polyclonal anti-NF 200 at a 1:150 dilution of a 9.87 mg/ml stock solution (Sigma Chemical Co., St. Louis, Missouri); (2) monoclonal antibody generated to mouse NF 200 (phosphorylated and nonphosphorylated forms; Clone N52) at a 1:150 dilution of the stock solution (Sigma Chemical Co., St. Louis, Missouri); (3) monoclonal anti-microtubule-associated protein-2 (anti-MAP-2) at a 1:2000 dilution of a 2 mg/ml stock solution (clone AP20; Boehringer Mannheim Corp., Indianapolis, Indiana); (4) monoclonal anti-tau microtubule-associated protein (anti-tau) at a 1:100 dilution of the 1 mg/ml stock solution (clone Tau 1; Boehringer Mannheim Corp., Indianapolis, Indiana); or (5) undiluted hybridoma supernatant to an anti-IFA antibody (Antibody to Intermediate Filament Antigen; Pruss et al., 1981) for 1 h at 4°C. Incubation was followed by biotinylated anti-rabbit or anti-mouse immunoglobulins and an avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, California) according to manufacturer's directions. Conjugates were visualized with a 4-chloro-l-naphthol/peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, Maryland).

**Results**

**Bodian silver stain**

The Bodian method was originally developed to stain neural components and is known to stain NF proteins specifically. Bodian silver stain was applied to tissue sections and to SDS polyacrylamide gels after electrophoresis. Neither method produced a visible reaction product (not shown).

**Light microscopic immunocytochemistry**

Figure 1 shows a transverse section of anemone tentacle stained with polyclonal antisem raised to NF 200. These antibodies exhibit wide species cross-reactivity (Chemicon International, Temecula, California, Sigma Chemical Co., St. Louis, Missouri). A large proportion of the nickel-enhanced immunoreactive product was localized to an area above the mesoglea known to contain a dense plexus
of nerve cell bodies and processes (C. DellaCorte, C. Fulenwider, D. Hessinger, and W.O. McClure, manuscript in prep). Immunolocalization was also seen within the gastrodermis, central to symbiotic zooxanthellae. Absorption of the antibodies with an extract of C. gigantea neurofilament-like protein removed this reactivity. Replacement of the primary, specific antibody with normal serum, or deletion of the secondary antibody, also eliminated the appearance of product.

**Electron microscopic immunocytochemistry**

Immunocytochemical localization within the tissues of C. gigantea was examined further by electron microscopy. With the chosen protocol, we were able to preserve antigenicity. Cytoskeletal morphology could not be fully maintained, however. Immunogold labeling was predominately localized within the processes of the neural plexus (Fig. 2) above the layer of myocytes. Immunogold labeling displayed the same relative pattern in all cellular neural compartments considered. No appreciable number of gold particles were observed over non-neural tissues. The specificity of labeling was assessed with controls: sections adjacent to those immunolabeled with anti-NF 200 were reacted with normal serum. The control sections lacked the labeling described above, or showed only extremely sparse and randomly scattered distribution of gold particles, demonstrating low background. These observations provide additional evidence that the immunoreactive material is located in the neurons of C. gigantea.

**Gel electrophoresis and Western blotting**

To further demonstrate the existence of neurofilament-like proteins in this species, we probed Western blots of Triton-insoluble proteins with several specific immunological probes. Polyclonal NF 200 antiserum revealed two bands of apparent molecular weights 156,000 and 74,000 daltons (Fig. 3). A similar pattern (not shown) was found with a monoclonal antiserum known to react with an epitope present on both the phosphorylated and non-phosphorylated forms of the polypeptide in the tail domain of the NF-H subunit.

Western blots of Triton-insoluble material from C. gigantea were also probed with monoclonal antibodies to the microtubule-associated proteins, anti-MAP2 (Fig. 4) and anti-tau (Fig. 5), and with a monoclonal antibody (anti-IFA: Pruss et al., 1981) known to bind to most of the defined major intermediate filament proteins. MAP2 isolated from rat brain consists of two major polypeptides (MAP2a and MAP2b) of similar molecular weights, approximately 275 kD, although proteolysis can result in a 35 kD domain and a major 240 kD domain (Vallee, 1980). Clear patterns of immunolabeling of the putative neurofilament bands (i.e., 156,000 and 74,000 daltons) extracted from C. gigantea are evident in both blots probed with antibodies to MAP2 (Fig. 4) and tau (Fig. 5), as are the expected high molecular weight MAP2 protein band and the multiple 55 kD to 62 kD tau bands (Cleveland et al., 1977a,b) present in the control tissues of the rat. In a similar fashion, the anti-IFA Western blot revealed two distinct bands of about 156 kD and 74 kD in the extract from the tissues of the sea anemone (Fig. 6).

**Discussion**

The presence of neurofilament-like proteins in the sea anemone C. gigantea was investigated using
Figure 3. Western blotting. Proteins were separated on a 4/20% gradient gel and visualized with a polyclonal antibody directed against NF 200. Two bands of MW, 156 kD and 74 kD were specifically labeled in the extract from the sea anemone Condylactis gigantea. Lane 1: molecular weight standards (MW,); Lane 2: anemone S2 fraction, 5.0 μg protein per lane; Lane 3: anemone S2 fraction, 9.5 μg protein per lane; Lane 4: anemone S2 fraction, 12.0 μg protein per lane; Lane 5: anemone S2 fraction, 15.0 μg protein per lane.

Homologous to those found in other organisms are present in this species. Immunological studies also reveal species-specific differences in the number and the molecular weight of neurofilament proteins. In birds and crocodilia, NF proteins appear to be directly homologous to the neurofilament triplet. Other reptiles express only a single high molecular weight subunit immunologically related to both NF-H and NF-M (Shaw et al., 1984; Shaw, 1991). Studies in fish demonstrate between one and four (or more) major subunits (Phillips et al., 1983; Lasek et al., 1985; Dahl et al., 1986). Neurofilaments in invertebrates, however, are more variable than their vertebrate counterparts. Two high molecular weight subunits, each about 160 kD, are found in the marine fan worm Myxicola infundibulum (Eagles et al., 1981). Squid contain neurofilament subunits of 60, 200, and 500 kD (Roslansky et al., 1980), although NF proteins with approximate weights of 60, 70, and 220 kD have recently been identified (Szaro et al., 1991; Way et al., 1992). Aplysia neurofilaments have apparent molecular weights of 60 kD and 65 kD. This nudibranch is the

several methods, including Bodian stain which binds an acidic, glutamate-rich region in the carboxy terminal of NF-L, NF-M, and NF-H (Autilio-Gambetti et al., 1981). This staining technique, used to identify neurofilament proteins in many species, did not produce positive results. But other data gathered during our study suggest that neurofilament-like proteins are present in C. gigantea.

A number of immunological studies have shown regional phylogenetic conservation of neurofilament proteins (Chiu et al., 1980; Autilio-Gambetti et al., 1981; Shaw et al., 1984). Immunological staining of Western blots of Triton-insoluble proteins from C. gigantea revealed 156,000 and 74,000 dalton molecular weight bands that correspond to those seen in published electrophoretic profiles. Explaining the presence of a low molecular weight band when using monoclonal and polyclonal antisera to NF-H is problematic, however. Many polyclonal and monoclonal antibodies reportedly recognize epitopes shared by NF-H and NF-M (Lee et al., 1987; Schmidt et al., 1987). Cross-reactivity between these subunits and NF-L is less well-known, although there is a report of simultaneous immunodetection of all three NF subunits (Cochard and Paulin, 1984). The presence of these immunoreactive proteins suggests that polypeptide regions

Figure 4. Western blotting. Proteins were separated on a 4/20% gradient gel and were immunoblotted with a monoclonal antibody generated to MAP2. In the protein fraction from Condylactis gigantea, two bands of approximate molecular weights 156 kD and 74 kD are found. Immunolocalization to protein fractions from control tissue show a large molecular weight subunit. A smaller, low molecular weight subunit, which may be due to proteolysis, is also present. Lane 1: molecular weight standards (MW,); Lane 2: rat brain cytosolic fraction (S1), 15 μg protein per lane; Lane 3: rat brain membrane fraction (S2), 15 μg protein per lane; Lane 4: anemone S1 fraction, 10 μg protein per lane; Lane 5: anemone S2 fraction, 9.4 μg protein per lane; Lane 6: anemone S3 fraction, 10 μg protein per lane.
only species investigated to date that does not express a subunit with a molecular weight greater than 100 kD and whose NFs lack an affinity for Bodian stain (Lasek et al., 1985). The presence of only two neurofilament-like proteins in C. gigantea may be a reflection of species-specificity.

More evidence for the presence of neurofilament-like epitopes was provided by absorption of the extract containing the neurofilament-like proteins with neurofilament antibodies and by immunoblotting with an antibody, anti-IFA, known to recognize a common antigenic determinant. In many species, anti-IFA has identified the presence of intermediate filaments, although immunoreactivity was not detected in the single species of Cnidaria investigated by others (Pruss et al., 1981; Bartnik et al., 1985; Shaw, 1991). Our immunoblot results with anti-IFA, however, suggest that C. gigantea does, in fact, contain the evolutionarily highly conserved epitope. Recognition of this sequence within the rod portion of the filament has, in many cases, been used to identify intermediate filaments (Shaw, 1991).

Microtubules are primary cytoskeletal elements that form polymers with neurofilaments in the nerve cells of many species (Wuerker and Palay, 1969; Hirokawa, 1982). These polymers contain a variety of microtubule-associated proteins (MAPs), including MAP2 and the closely related protein family, tau (Borisy et al., 1975; Weingarten et al., 1975; Cleveland et al., 1977a,b). Antibodies generated to MAP2 and tau cross-react with neurofilament proteins (Kosik et al., 1988; Shaw, 1991). Investigations suggest similarities in sequence and the presence of heavily phosphorylated regions as one explanation for the cross-reactive properties. Tau from rat brain contains four internal repeats consisting of a “Lys-Ser-Pro-Val” motif in the COOH-terminal domain similar to the numerous Lys-Ser-Pro (KSP) repeats found in neurofilament (Kanai et al., 1989; Shaw, 1991). The well-characterized Tau 1 antibody used in this study recognizes an amino terminus epitope located between Pro 189 and Gly 207. This sequence is suggested to be similar to the carboxy-terminus of NF-M (Kosik et al., 1988; Goedert et al., 1991). The antibody is not known to react, however, when Ser 199 and Ser 202 are phosphorylated (Riederer and Binder, 1994). The MAP2 an-
tibody, clone AP20, has only recently been mapped to a carboxy terminus encompassing amino acids 997-1332 (L. Binder, pers. comm.). A region suggested to contain sequences closely related to those found in NF-H, NF-M, and tau proteins (Shaw, 1989). The presence of these conserved regions suggests evolutionary relationships and may account for the immunological similarity seen in a number of studies, including ours.

Considering the well-documented description of a diffuse nerve net in cnidarians, we were surprised to find little immunostaining throughout the epidermis and gastrodermis of the tentacle using anti-NF200. Most of the reaction product was localized within an epidermal neural plexus and, to a lesser extent, to components within the gastrodermis. This pattern was reversed when tissues were immunostained with a polyclonal antiserum generated to NF 150 (C. Dellacorte, unpub. ob.). Selective reactivity of the antisera (i.e., immunodetection of specific subsets of neural components) was not expected but may be the result of several factors including cell size, cell type, presence or lack of post-translational modifications, or the cross-reactivity of the antisera. Many regions in mammalian nervous systems show strong NF staining for some neuronal cell types and partial or complete absence of immunoreactivity in others. This is particularly true for small neurons (Sharp et al., 1982; Trojanowski et al., 1986). In addition to other factors, the type and size of cells in the nervous system of *Condylostoma gigantea* may affect the presence and the extent of immunoreactivity in this invertebrate.

The present data suggest that neurons in this anthozoan may contain proteins with molecular properties similar to those of higher species. Although various assumptions about these neurofilament-like proteins may be made, no definitive conclusions can yet be drawn. Knowledge of the primary structure of these early metazoan proteins will provide insight into the functional constraints of protein sequences during evolution.

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**Literature Cited**


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