A Novel Intermediate Filament-associated Protein, NAPA-73, That Binds to Different Filament Types at Different Stages of Nervous System Development

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Abstract. The antigen recognized by the E/C8-mono- 
clonal antibody is expressed in various avian embry- 
onic cell types known also to express neurofilament 
(NF) immunoreactivity. To determine whether the 
E/C8-antigen corresponds to any of the known NF 
components, we compared their subcellular locations, 
immunocross-reactivities, and electrophoretic behav- 
iors. We found that the E/C8-antibody binds to NF 
bundles in electron microscope preparations of neu- 
rons, but does not correspond to any of the known 
NF proteins by immunological or electrophoretic cri- 
teria. Immunoadsorption with the monoclonal anti- 
body resulted in co-purification of a 73,000-D protein 
with one of the known NF proteins in homogenates 
from 20-d embryonic chick brains, but with vimentin 
intermediate filament protein in similarly prepared 
homogenates from 4-d embryonic chicks. We suggest 
that the E/C8-antigen is an intermediate filament- 
associated protein that binds to different filament 
types at different stages of development. We have 
named it NAPA-73, an acronym for neurofilament- 
associated protein, avian-specific, 73,000 D, on the 
basis of its binding specificity in mature neurons.

A number of neuronal markers appear early in the devel- 
opment of the vertebrate nervous system (e.g., Smith 
et al., 1979; Barald, 1982). In birds, one of the earliest 
markers is an antigen recognized by the monoclonal antibody 
E/C8 (Ciment and Weston, 1982). This antigen can be de- 
dected in the embryonic neural tube before the first neurob- 
lasts are reported to undergo their terminal cell division (Hol- 
llyday and Hamburger, 1977). E/C8-immunoreactivity is also 
detected in a subpopulation of migrating and dividing neural 
crest cells (Ciment and Weston, 1985)—the developmental 
ancestors of the peripheral nervous system (Le Douarin, 
1982; Weston, 1982).

Similar immunocytological observations have been made 
using antisera against various components of the neurofila- 
ment (NF) complex—the intermediate (10 nm) filament type 
found in neurons. In both the neural tube (Tapscott et al., 
1981 a; Bennett and DiLullo, 1985) and in migrating neural 
crest cells (Payette et al., 1984), NF-immunoreactivity appears 
at roughly the same time as E/C8-immunoreactivity (Ciment 
and Weston, 1982, 1985). Moreover, both of these antigens 
are intracellular and protease-sensitive (Ciment and Weston, 
1982; Nixon, 1983).

Because of these common immunocytological properties, 
it is possible that the E/C8-antigen corresponds to some 
component of the NF complex. Neurofilaments are believed 
to consist of three proteins, which in birds are 70,000, 
160,000, and 180,000 D (NF-L, NF-M, and NF-H, respec- 
tively [Willard and Simon, 1983; Tapscott et al., 1981 b]). To 
test whether the E/C8-antigen corresponds to any of these 
proteins, we compared their subcellular location, apparent 
molecular weights, and immunological properties. We report 
here that the E/C8-antigen is associated with bundles of NFs 
in post-mitotic neurons, as seen in the electron microscope, 
but that it does not correspond to any of the known triplet 
proteins by the other criteria.

Materials and Methods

Cell Culture

Dorsal root ganglia (DRG) were dissected from 9-11-d chicken embryos and 
dissociated with trypsin (0.1%). Cells were plated on polyornithine-treated glass 
coverslips in 35-mm plastic Petri dishes (No. 1008, Falcon Labware, Oxnard, 
CA) for immunofluorescence, and on polyornithine-treated formvar films on 
gold electron microscopy grids for whole mount immunoelectron microscopy. 
 Cultures were grown for 24-48 h in medium consisting of F-12 nutrient mixture 
(Gibco, Grand Island, NY) and heart conditioned medium (1:1, vol/vol) 
(Letourneau, 1983). Nerve growth factor (a gift from Dr. Eric Shooter) was 
added to culture medium at a concentration of 10 ng/ml.
**Immunoreagents**

Rabbit antisera against chicken NF-L and NF-M proteins were gifts from Drs. Bruce Granger and Elias Lazerides. Antiserum against chicken vimentin was a gift from Dr. Robert Goldman. Other serological products were purchased from Cappel Laboratories (Cochranville, PA).

**Immunofluorescence**

DRG cultures were extracted for 20 min at room temperature with PHEM buffer (Schliwa and van Blerkom, 1981) containing 0.2% Triton X-100, and then fixed with 4.0% paraformaldehyde in PHEM buffer for 20 min at room temperature. After rinses in phosphate-buffered isotonic saline (PBS), the coverslips or grids were incubated for 15 min at 37°C in buffer A consisting of PBS with 5 mg/ml bovine serum albumin (BSA) and 0.2% Triton X-100. The cultures were then rinsed in PBS, incubated for 40 min at 37°C in a 1:50 dilution of E/C8-hybridoma conditioned medium in buffer A, rinsed in PBS, incubated 15 min again in buffer A, and then incubated with a 1:100 dilution of rhodamine-conjugated rabbit anti-mouse immunoglobulin in buffer A for 30 min at 37°C. As controls, other IgM monoclonal antibodies were substituted for E/C8.

For double labeling experiments, rabbit anti-NF-M or anti-vimentin antisera (1:100 dilutions) were included along with E/C8-conditioned medium in the primary antibody incubation mixture. After washes in PBS, secondary incubations were performed using fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit immunoglobulins. Non-immune serum was substituted as controls for anti-NF or anti-vimentin antisera. Cultures were rinsed with PBS, then water, and mounted in Elvanol. These preparations were viewed through a 63× planapochromat objective with a Zeiss IM microscope (Carl Zeiss, Inc., Thornwood, NY) illuminated by an HBO 100-W mercury bulb. Photomicrographs were taken on Tri-X film with exposures of 10-45 s.

**Electron Microscope Immunocytochemistry**

After incubation with E/C8-hybridoma conditioned medium and rabbit anti-immunoglobulin, the electron microscopy grids were rinsed in buffer B consisting of 20 mM Tris (pH 8.2), 0.9% NaCl, and 1.0 mg/ml BSA. The grids were then incubated for 30 min in a 1:2 dilution of 10-nm colloidal gold absorbed with goat anti-rabbit immunoglobulin (Janssen Pharmaceutical, Inc.), rinsed in buffer B containing 10 mg/ml BSA, and fixed with 1% glutaraldehyde in PBS for 15 min. Immunogold staining with anti-NF-M antibody was performed by initial incubation of cytoskeletal preparations with a 1:50 dilution of anti-NF-M, followed by rinses in buffer B, and subsequent incubations with colloidal gold absorbed with goat anti-rabbit immunoglobulin. The fixed cultures were then rinsed in PBS, stained with tannic acid (2%, 20 min), and uranyl acetate (2%, 20 min), dehydrated in alcohol, and critical point dried, as described (Letourneau, 1983). The grids were examined with a JEOL 100CX electron microscope (JEOL USA, Peabody, MA). In control cultures, primary antibodies were omitted from the initial incubations.

**Purification of NF-M**

The method of Granger and Lazarides (1983) was used first to purify an intermediate filament-enriched fraction. This preparation was then subjected to electrophoresis on an SDS (10%) polyacrylamide gel, and visualized using the silver staining method of Morrissey (1981).

**Purification of NAPA-73**

A modification of the method of Gallatin et al. (1983) was used, as follows. Purified E/C8-antibody (in 0.9% NaCl) was incubated overnight at 4°C in 96-well polyvinyl chloride plates (U-shaped, Cooke). Plates were then washed with water and incubated with a 1.0% BSA solution in PBS (3 h at room temperature) to block nonspecific binding sites. The plates were then washed in PBS with 1.0% Triton X-100 and stored in water until use.

Homogenates were prepared from freshly dissected tissue by repeated passage through 18- and 26-gauge needles on disposable syringes, followed by centrifugation (10 min) in an Eppendorf microfuge to remove particulate material. Homogenization buffer consisted of sodium phosphate (100 mM, pH 7.5), EDTA (5 mM), phenylmethylsulfonyl fluoride (0.5 mM), aprotinin (100 KIU/ml), SDS (0.1%), Triton X-100 (5%), and urea (0.25 M). Homogenates containing equivalent amounts of protein were added to the wells, and the plates were incubated overnight at 4°C. The plates were then washed exhaustively with water, PBS plus Triton X-100 (1%), 10× PBS, and then water. Antigen was eluted by adding 5.0% SDS, and the eluates were boiled for 3 min. Proteins in these eluates were subjected to electrophoresis in an SDS (10%) polyacrylamide gel, and visualized using the silver staining method of Morrissey (1981).

**Immunoblotting**

Protein bands were transferred to nitrocellulose sheets using a Transblot apparatus (Bio-Rad Laboratories, Richmond, CA), and visualized using the India Ink method of Hancock and Tsang (1983). The sheets were incubated overnight at 4°C in a solution of 1.0% BSA, 1.0% Ficoll (400,000 D, Sigma Chemical Co., St. Louis, MO), 1.0% polyvinylpyrrolidone (40,000 D, Sigma Chemical Co.) in PBS in order to block nonspecific sites, and then incubated with rabbit antiserum against vimentin, NF-L, or NF-M (1:100-500 dilutions in PBS with 1.0% BSA, 1 h, room temperature). After washing in PBS, sheets were incubated with anti-rabbit IgG antisera (1:200 dilution), rinsed, incubated with 111I-protein A (1.0 μCi/ml in PBS with 1.0% BSA; 9.5 mCi/mg protein A, New England Nuclear, Boston, MA), and washed thoroughly. Autoradiography was performed using presensitized x-ray film exposed for 4-7 d. Labeled protein bands on the autoradiographs were identified by comparison with the India Ink-stained protein bands on the nitrocellulose paper.

**Results**

**Immunofluorescence**

E/C8-antibody stained both the cell bodies and neurites of DRG neurons but not flat non-neuronal cells in the same cultures (Fig. 1 A; also compare A and B of Fig. 2). In some neuronal somata, E/C8-mediated fluorescence appeared as a perinuclear network of filamentous material (not shown). Along neurites, staining often appeared as longitudinally oriented fibers fraying out from the Triton X-100–extracted neurites (Fig. 1 B, arrows). Neither neurons nor non-neuronal cells bound the fluorescent second antibodies alone (not shown).

The staining patterns with E/C8-antibody are similar to studies using antisera against components of the neuronal cytoskeleton, including the NF-M protein (Raju et al., 1981) and vimentin (Jacobs and Thomas, 1982; Jacobs et al., 1982). To compare the patterns directly, we stained DRG cultures with antibodies against the E/C8-antigen (Fig. 2, B and D) and either NF-M protein (Fig. 2 A) or vimentin (Fig. 2 C). Although there were some minor differences in the NF-M and E/C8 staining patterns (e.g., in the degree of nuclear staining), in general, the same cells expressed both immunoreactivities. These cells are presumably neurons. In

![Figure 1. E/C8-mediated immunofluorescence staining of DRG neurons in culture. (A) Triton X-100–treated culture. (B) Triton X-100 and high salt–treated culture. Note the immunostaining of neurons and neurites, but not non-neuronal cells, in both conditions. Also note the staining of thin fibers in the high salt–treated cultures (arrows). n, neuron; g, non-neuronal cell. Bar (for A and B), 40 μm.](image-url)
contrast, both non-neuronal cells and neurons stained with anti-vimentin antibody (Fig. 2C).

Extraction of microtubules with a high salt buffer (Schliwa and van Blerkom, 1981) had little effect on either E/C8- or NF-M-immunostaining (not shown). Confirmation that microtubules were dissociated by this high salt treatment was

Figure 2. Double immunolabeled cultures of DRG neurons. A and B are the same microscopic field showing NF-M and E/C8-immunoreactivities, respectively. C and D are a similar culture showing vimentin and E/C8-immunoreactivity, respectively. Note the coincidence of E/C8- and NF-M-immunostaining. g, non-neuronal cell. Bar (for A–D), 20 μm.

Figure 3. Immunogold labeling of bundles of 10-nm intermediate filaments in cultured DRG cells. (A) E/C8-mediated decoration of a neuron, showing the presence of immunoreactivity in bundles, but not individual strands, of NFs. (B) E/C8-mediated decoration of a neuron atop a non-neuronal cell. Note again the absence of colloidal gold labeling of the individual filament strands (presumably gliofilaments). (C) Anti-NF-M-mediated decoration of a neuron, showing labeling of individual strands. (D) Control preparation incubated without a primary antibody. Note the low background decoration by colloidal gold particles (arrows) and the lack of microtubule profiles. (E) Control preparation before immunogold labeling procedures, showing the naked 10-nm NFs and 2-nm filaments (arrows). Bar: A and B, 160 nm; C, 300 nm; D, 500 nm; E, 150 nm.
obtained both by immunofluorescence methods, using antisera against tubulin (Letourneau and Ressler, 1984; not shown), and by examining extracted cells in the electron microscope (see Fig. 3 D, below).

**Electron Microscope Immunocytochemistry**

In the electron microscope, colloidal gold labeling revealed E/C8-immunoreactive material associated almost exclusively with bundles of 10-nm filaments (Fig. 3A), whereas antisera to NF-M mediated the decoration of both bundles and individual 10-nm filaments (Fig. 3C). In control cultures incubated with only secondary antibodies and the colloidal gold reagent, there was minimal background decoration (Fig. 3 D, see arrows).

In addition to the presence of 10-nm filaments in many of these preparations, there were also small numbers of short 2-nm filaments attached at one or both ends of 10-nm filaments (Schwila and van Blerkom, 1981) (Fig. 3E, arrows). These filaments were resistant to the extraction conditions used here, and displayed no E/C8-mediated decoration (not shown).

**Molecular Characterization of the E/C8-Antigen**

The above results suggest that the E/C8-antigen is a component of the NF complex in neurons. To determine which, if any, of the known NF components is being recognized by this antibody, the E/C8-antigen was purified using immunofinity methods, and its molecular weight compared with published values of the NF proteins (Tapscott et al., 1981b). For these experiments, tissues were taken from relatively young (4–7-d-old) chicken embryos to minimize the number of E/C8-positive nerve fibers that later contaminate control tissues in older embryos.

Fig. 4 shows an SDS polyacrylamide gel containing proteins immunoabsorbed from these tissue homogenates. Two distinct bands—one at 73,000 (73K) and another at 54,000 (54K) D—were immunoabsorbed by E/C8-antibody from

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**Figure 4.** SDS polyacrylamide gels of E/C8-immunopurified proteins from various tissues of 4-d chicken embryos. Lanes T (nos. 1, 4, and 7) are total proteins in the homogenates; lanes + (nos. 2, 5, and 8) are proteins eluted off E/C8-antibody-coated wells; lanes − (nos. 3, 6, and 9) are from control wells in which only BSA was adsorbed to the wells. Note the bands at 73,000 (NAPA-73) and 54,000 D in the eluate from homogenates of E/C8-immunoreactive embryonic tissues (lanes 2 and 3), and the lack of corresponding bands from eluate from homogenates of non-immunostaining tissue (lane 8).
tissues known to express E/C8-immunoreactivity (e.g., brain [lane 2] and posterior branchial arches [lane 5]; Ciment and Weston, 1982, 1983), but not from tissues known to lack immunoreactivity, such as embryonic liver (lane 8). There is no detectable binding of these proteins when E/C8-antibody is not present (lanes 3 and 6), or when other IgM monoclonal antibodies are substituted for E/C8-antibody (not shown).

The 73,000-D band in the E/C8-immunoadsorbed preparation has an electrophoretic mobility that is clearly different from that of the NF-L component (Fig. 5A). Moreover, anti-NF-L antibody binds to the NF-L bands of lanes 2 and 3, but not to the 73K E/C8-protein (lanes 1 and 1') (Fig. 5B). The converse experiments, where E/C8-antibody is used to probe immunoblots, were not successful, presumably because the E/C8-antigen loses immunoreactivity after electrophoresis and blotting.

The protein bands found to co-purify with the 73,000-D band differed in the preparations immunopurified using E/C8-antibody from embryos of different ages. From 4-d embryonic chick heads, a 54,000-D band was found to co-

Figure 5. Non-equivalence of NAPA-73 and NF70 by biophysical and immunological criteria. (A) Silver stained SDS (10%) polyacrylamide gel of proteins purified from 20-d embryonic chicken brains. (B) x-ray autoradiographs of an anti-NF70 immunoblot from a gel similar to that in A. Lanes 1 and 1' correspond to immunopurified NAPA-73 (middle arrowhead at left); lanes 2 and 3' correspond to partially-purified NF70 (lower arrowhead at left); lanes 2 and 2' correspond to a mixture of both NAPA-73 and NF70 preparations. The upper arrowhead at left corresponds to NF160. Note in B that antiserum against NF70 does not recognize NAPA-73 or NF160.

purify with the 73,000-D band (Fig. 4, lane 2), whereas 20-d embryonic chick brains yielded little of the 54,000-D band, but substantial amounts of a 160,000-D protein (Fig. 5, lane 1). The 54,000-D band binds antibodies to vimentin (Fig. 6, lane 2), and the 160,000-D band binds antibody against NF-M (Fig. 6, lane 6).

Discussion

The E/C8-Antigen Is Associated with Bundles of Intermediate Filaments in the Cytoskeleton of Neurons and Other Cell Types

For a number of reasons, the E/C8-antigen is likely to be a component of the NF complex of the cytoskeleton in various cell types. In previous work (Ciment and Weston, 1982, 1983, 1985), we showed that E/C8-immunoreactive material is present in a number of avian tissues known to contain NFs, such as the neural tube (Tapscott et al., 1981a, b; Bennett and DiLullo, 1985) and the embryonic branchial arches (Payette et al., 1984), and occurs at about the same developmental stage in these cells as NF-immunoreactivity (Tapscott et al., 1981a, b; Ciment and Weston, 1982, 1985; Payette et al., 1984; Bennett and DiLullo, 1985). We have now shown at the ultrastructural level that the E/C8-antigen is associated with NF profiles in individual neurons. E/C8-immunoreactivity is associated almost exclusively with bundles of NFs, rather than with individual 10-nm strands (Fig. 3, A and B). In contrast, NF-M immunoreactive material
was associated with both bundles of filaments and individual strands (e.g., Fig. 3C). We doubt that the lack of decoration of single strands by E/C8 was an artifact introduced during processing for the electron microscope, because even when such strands formed a dense network, such as when neuronal cells sat atop non-neuronal cells, there was no decoration of individual strands (Fig. 3B).

The E/C8-Antigen Is Probably a 73,000-D, Intermediate Filament–associated Protein

When homogenates of neural tissues from embryos of various ages were used as the source of E/C8-antigen, only pairs of protein bands were co-purified—one pair from early (4-d) embryonic tissue at 54,000 and 73,000 D, and another pair from older (20-d) embryonic brains at 73,000 and 160,000 D. This co-purification pattern can be explained in two ways. First, the three proteins might share the E/C8-epitope. Alternatively, one of these proteins may contain the epitope and remain tightly bound during purification to the other proteins present in the homogenate.

Since the 54,000- and 160,000-D proteins were identified in immunoblots as vimentin and NF-M, respectively, and since neither vimentin nor NF-M protein are present in all tissues known to express E/C8-immunoreactivity, the common protein band at 73,000 D is likely to be the E/C8-antigen. Moreover, since the molecular weight of this protein does not correspond to published values for the NF proteins (Tapscott et al., 1981b), yet is associated with the NF complex in situ, it seems likely to be a novel intermediate filament–associated protein. We propose that this novel protein be called NAPA-73, an acronym for neurofilament-associated protein, avian-specific, 73,000 D on the basis of its binding specificity to mature neurons.

The E/C8-Antigen NAPA-73 Is Associated with Different Filament Types at Different Stages of Nervous System Development

The fact that NAPA-73 co-purifies with vimentin and NF-M from homogenates of 4-d and 20-d embryonic chicks, respectively, suggests that NAPA-73 normally associates with different intermediate filament types at different stages of development. The time of switching from vimentin to NF-M, moreover, is consistent with observations that neural crest cells and proliferating neuroblasts of the chick neural tube express vimentin immunoreactivity, whereas the neuroblasts begin to express NF-M immunoreactivity just before terminal mitosis (Tapscott et al., 1981a). Although it seems likely that NAPA-73 immunoreactivity is expressed throughout the entire period, double-labeling experiments for NAPA-73, vimentin, and NF-M will be needed to clarify this point.

As discussed above, we have been unable to detect E/C8-mediated staining of isolated protein bands in gels or immunoblots. We have previously reported, moreover, that E/C8-mediated staining of tissues is highly vulnerable to histological fixation and other experimental perturbations (Ciment and Weston, 1982). If NAPA-73 normally associates with other intermediate filament types, its anomalous immunoreactive properties may be related to these associations. Thus, it is tempting to speculate that NAPA-73 may require specific association with other cytoskeletal components in order to maintain the E/C8 epitope in an immunoreactive configuration (see also Danto and Fischman, 1984).

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