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The Single Neurofilament Subunit of Lamprey May Need Another Element for Filament Assembly

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ABSTRACT

Regenerating axon tips in transected lamprey spinal cord contain dense accumulations of neurofilaments (NFs), suggesting that NFs may play a role in the mechanism of axonal regeneration. Compared with heteropolymeric assemblies of NF triplet proteins in mammals, NF in lampreys has been thought to contain only a single subunit (NF180). This would imply that NF180 self-assembles, which would be important for manipulating its expression in studies of axonal regeneration. In order to study the possible role of NF in process outgrowth and to determine whether NF180 can self-assemble, its gene was transfected into mammalian and fish cell lines that either contain or lack vimentin. In transfected NIH3T3 cells, NF180 was poorly phosphorylated and its expression did not alter the length or number of cell processes. Nor did it appear to form typical intermediate filaments, suggesting that it may not self-assemble. NF180 also did not form typical filaments in SW13cl cells that either possessed or lacked vimentin, nor in transfected fish cells that were cultured at 18°C. In vitro, NF180 could not self-assemble but interacted with NF-L to interrupt its self-assembly. When cotransfected with rat NF-L into SW13c1.2vim$^-$ cells, NF180 did form thick, rod-like filamentous structures on immunofluorescence. More typical NFs were observed when NF180 was cotransfected with both NF-L and NF-M. Thus, NF180 cannot self-assemble but appears to require one or more additional elements for incorporation into NFs. J. Comp. Neurol. 471: 188–200, 2004.

Indexing terms: NF180; self-assembly; vimentin; cytoskeleton; axonal regeneration

Lamprey spinal axons regenerate following spinal cord transection (Selzer, 1978; Cohen et al., 1988). However, the mechanisms underlying elongation of the regenerating growth cones are not clear. In cultured embryonic neurons, growth cones lack NFs and migrate rapidly via a pulling action of filopodia and lamellipodia that is generated by complex interactions among actin microfilaments, myosin, and microtubules (Stroh et al., 1990; Forscher et al., 1992; Lin and Forscher, 1993). By contrast, growth cones in regenerating CNS of lamprey appear to migrate slowly, lack filopodia and lamellipodia, have little F-actin, but are densely packed with highly phosphorylated NFs (Lurie et al., 1994; Hall et al., 1997). Moreover, regenerative ability among reticulospinal neurons was positively correlated with the ability to re-express NF mRNA after transient downregulation (Jacobs et al., 1997). It was therefore postulated that the assembly and transport of

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NFs into the growth cone may contribute a protrusive force that contributes to regeneration (Pijak et al., 1996). This has led us to investigate the assembly properties of lamprey NF.

NFs are neural-specific intermediate filaments (IF) that comprise most of the cytoskeleton of the axon, where they appear to provide mechanical support and mediate increased diameter. However, the functions of NFs are not fully understood. The function of NFs in vertebrate neurons has been explored with the aid of transgenic and knockout mice (Zhu et al., 1997; Elder et al., 1998; Hirokawa and Takeda, 1998), but because of the triplet nature of mammalian NF, its role in regeneration has been difficult to determine. Of the three mammalian NF subunits, only the low molecular weight subunit (NF-L) can self-assemble into filaments in vitro (Geisler and Weber, 1981), while the middle and high molecular weight subunits (NF-M and NF-H) require the presence of NF-L to form normal filaments (Leung and Liem, 1996). In vivo, however, all three NF subunits from rat are incapable of self-assembly when expressed in cells lacking a preexisting IF network (Ching and Liem, 1993; Lee et al., 1993), although human NF-L has been reported to self-assemble in vivo (Carter et al., 1998). NF-L coassembles with either NF-M or NF-H into filamentous structures in transfected cells, but NF-M cannot form filaments with NF-H. By contrast with mammalian NFs, thus far only one NF subunit, NF180, has been identified in the lamprey (Pleasure et al., 1989), so it has been postulated that NF180 can self-assemble into filaments. If this were true then it would greatly simplify analysis of the role of NFs in the regeneration of lamprey CNS axons. However, the ability of NF180 to self-assemble has not been well studied. Hall et al. (2000) demonstrated that transfecting NF180 into neuroblastoma (NB2a) cells which were induced to differentiate by application of dibutyryl cyclic AMP (db cAMP) resulted in the incorporation of NF180 into NFs and production of somatic swelling by 3 days posttransfection. However, since NB2a treated with db cAMP normally contain bundles of NFs (Shea et al., 1985), it is possible that in the transfected cells NF180 either coassembled with other NF subunits or merely decorated preformed NFs. The purpose of the present study was to determine whether NF180 is capable of self-assembly by transfecting it into cultured cells (both mammalian and fish-derived) that either contain or lack a preexisting IF network and by testing the ability of NF180 to form NFs through homopolymerization in vitro.

MATERIALS AND METHODS

Constructions of NF180

A single NF subunit (NF180) gene of lamprey was cloned from a lambda-gt11 library constructed from the brains of larval wildtype lamprey (*Petromyzon marinus*, Jacobs et al., 1995). Clone LIF22 contains the ATG translation start codon and two-thirds of the NF180 sequence. LIF13 overlaps the 3′ end of LIF22 and contains the TGA stop codon and polyadenylation signal. A full-length NF180 was created from the two clones LIF13 and LIF22 by using a unique restriction enzyme (Stu I, at nucleotides 2436) site in the overlapping region. The NF180 cDNA was ligated into Hind III/Xba I sites of pRC/CMV, a eukaryotic expression vector (Invitrogen, La Jolla, CA), to form NF180-pRC/CMV. A control construct was created by ligating LIF 22 into the pRC/CMV vector in an antisense orientation. In order to allow rapid identification of NF180-positive cells, permit labeling manipulations with other IFs and distinguish transfected from endogenous NF180 in future in vivo lamprey experiments, NF180 was inserted to pEGFP-C3 (ClonTech, Palo Alto, CA) in the correct reading frame (NF180-pEGFP-C3). In order to characterize the NF180 for self-assembly or coassembly with other IF in vitro, the full-length cDNA of NF180 was subcloned into a bacterial expression vector pET 11b (NF180-pET 11b, Novagen, Madison, WI) by PCR strategy. The constructs were purified with plasmid purification kit (Qiagen, Chatsworth, CA).

Antibodies

Monoclonal antibodies (mAb) to NF180 were generated by using the Triton X-100 insoluble cytoskeletal fractions obtained from lamprey spinal cords and identified, characterized by ELISA, Western blotting, and immunocytochemistry (designated as LCMs, Merrick et al., 1995). A total of seven NF-specific LCMs were identified, of which two, LCM3 and LCM39, were used for this experiment. LCM3 is a phosphate-independent antibody binding to the core of NF180. LCM39 is a phosphate-independent antibody binding to the tail of NF180. Two additional anti-NF antibodies, RMd015 and RMO62, were provided by Dr. Virginia Lee (Dept. of Pathology and Laboratory Medicine, University of Pennsylvania). RMd015 and RMO62 are mAbs specific for dephosphorylated and phosphorylated epitopes, respectively, in the rat, and which were previously found to bind lamprey NF180 and label lamprey spinal axons by immunohistochemistry (Pleasure et al., 1989). MAbs to vimentin and α-tubulin was obtained commercially (Sigma, St. Louis, MO, V6630 and DM1A). The anti-NF-L mAb was from Sigma and anti-GFP polyclonal antibody from ClonTech. Polyclonal antibodies for rat NF-L and NF-M were described previously (Beaulieu et al., 1999).

NF180 expression in cultured cells

Mouse fibroblast (NIH3T3) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO_2. Passaged cells were grown on sterile glass coverslips coated with 0.01% of poly-L-lysine (Sigma) in 35 mm dishes to 80% confluence on the day before transfection. Two μg of NF180-pRC/CMV were introduced into cells using the Lipofactmine reagent (Gibco BRL, Gaithersburg, MD). The cells were harvested 48 hours after transient transfection for either immunostaining or Western blotting. In order to observe cell phenotype changes caused by expressing NF180, a stably transfected cell line was established by subculturing transfected cells on the selecting medium containing 600 μg/ml of G418 (Gibco BRL) for 48 hours after transfection. The G418-resistant colonies were screened for NF180 expression by both immunocytochemical staining and PCR. NIH3T3 cells are known to contain vimentin, which could coassemble with NFs (Chin and Liem, 1990; Sun et al., 1997). Therefore, in order to determine whether NF180 is capable of self-assembly into NFs, expression of NF180 was also induced in the human adrenal carcinoma cell lines that either contain (SW13cl.1Vim$^+$) or lack (SW13cl.2Vim$^-$) preexisting cytoplasmic intermediate filaments. (SW13cl cells

were a gift from Dr. Robert Evans, Department of Pathology, University of Colorado, Health Sciences Center). SW13cl.2Vim$^-$ cells were also used to test the heterogeneous assembly between NF180 and mammalian neurofilament subunits (NF-L, NF-M, NF-H) by cotransfection as described previously (Beaulieu et al., 1999). Since assembly of fish vimentin is temperature-sensitive (Herrmann et al., 1993, 1996), we expressed NF180 in the above cells at 37°C, room temperature (25°C), and 15°C. We also included three fish cell lines (obtained from American Type Culture Collection), RTG-2 (derived from rainbow trout gonadal cells), CHES-214 (derived from Chinook salmon embryos), and RTH-149 (derived from rainbow trout hepatoma). These cells were cultured at 18°C. The SW13cl cells were grown in DMEM/F12 medium (Gibco BRL) supplemented with 5% FBS (pH 7.4) and the fish cells were grown in minimal essential medium (MEM) supplemented with 10% FBS in a humidified incubator with 5% CO_2. The transfection of NF180 was done as above.

Quantifying cell morphology

The images of immunostained NF180-transfected cells were captured using a CCD video camera (NEC TI-24A) mounted on a Zeiss Axioskop microscope. As a control, nontransfected cells grown in the same conditions were stained with α-tubulin antibody. Process lengths were measured from the nuclear edge to the tips of the processes using a computer-based video image analysis system (JAVA; Jandel, San Rafael, CA). The numbers of processes in each cell (process length > diameter of nucleus only) were counted. The data were analyzed for statistical significance using Student's *t*-test with Bonferroni correction for multiple comparisons, where appropriate.

Immunocytochemistry

Cells grown on coverslips were rinsed in phosphate-buffered saline (PBS, pH 7.4) and fixed in 2% paraformaldehyde in PBS for 20 minutes at room temperature followed by 3 minutes in cold acetone at –20°C. After several washes with PBS the cells were treated with PBS containing 3% bovine serum albumin (BSA) for 30 minutes and incubated with primary antibodies overnight at 4°C. Then cells were washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. The samples were developed with a metal enhanced diaminobenizidine (DAB) substrate kit (Pierce, Rockford, IL) for 15 minutes. The coverslips were then dehydrated, cleared, inverted, and mounted onto glass microscope slides with Permount.

In order to view the filament structures with light microscopy, immunofluorescence staining was performed. After washing with PBS the transfected cells were fixed and extracted in cold methanol at –20°C for 10 minutes. The cells were then washed, blocked, and incubated with mAb LCM3 to NF180. After overnight incubation the cells were washed and incubated with rhodamine-conjugated secondary antibody for 1 hour at room temperature. For double-labeling experiments, GFP-tagged NF180 was detected using an anti-GFP polyclonal antibody and FITC-conjugated secondary antibody, while vimentin or NF-L was detected using anti-vimentin or anti-NF-L mAb and rhodamine-conjugated secondary antibody. The coverslips were washed and mounted onto glass microscope slides with Fluoromount-G (Fisher Scientific, Pittsburgh, PA). Filaments were viewed on a fluorescence microscopy with a 100× oil objective lens (Zeiss Axioskop).

Immunoblotting

In order to verify the size of NF180 protein that was expressed in cell culture, the cells were harvested 48 hour after transient transfection and lysed directly in SDS sample buffer containing 0.125M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol and then boiled for 5 minutes prior to being loaded on the gels. The proteins were separated in 7.5% acrylamide SDS gel and electrophoretically transferred onto nitrocellulose membranes using a Bio-Rad (Hercules, CA) transblot apparatus. Then the membranes were preincubated in 5% nonfat dry milk in Tris-buffered saline (TBS, pH 7.4) for 30 minutes to deter nonspecific interactions of the antibody. The blots were incubated with NF180 mAbs, LCM3 and LCM39 (1:100), overnight at 4°C, washed extensively with 0.1M TBS containing 0.1% Tween-20, and incubated with HRP-conjugated antimouse IgG secondary antibody (Santa Cruz Biologicals, Santa Cruz, CA) for 1 hour. After washing with 0.1M TBS for 30 minutes, immunoreactive proteins were visualized by development with DAB substrate in the presence of hydrogen peroxide. As a positive control, NF-enriched lamprey CNS cytoskeletal proteins, prepared as described elsewhere (Pleasure et al., 1989), were also included.

NF180 expressed in *E. coli* was extracted as described below. Identification was verified as above except for detection by chemiluminescence (Pierce) and visualized by a 2-minute exposure to film.

In situ hybridization and Northern blotting

Digoxigenin-labeled cRNA riboprobes were constructed from a plasmid containing NF180 cDNA. In vitro transcription was performed with an RNA transcription kit (Stratagene, La Jolla, CA) as recommended by the manufacturer except for the inclusion of digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, IN) in a 35:65 ratio with unlabeled UTP. The transcripts were fragmented by incubation with 0.1M sodium carbonate at 65°C for 60 minutes and precipitated with ethanol. The transfected cells grown on coverslips were washed in DEPCed PBS and fixed with 4% paraformaldehyde for 30 minutes. The coverslips were hybridized to digoxigenin-labeled probes overnight at 55°C as described previously (Swain et al., 1994).

Total RNA was isolated from NF180-pRC/CMV-transfected NIH3T3 cells using Trizo reagent (Gibco BRL). Isolated RNA (20 μg) was then electrophoresed through a 1% formaldehyde agarose gel and transferred onto nitrocellulose membrane by capillary elution. The blot was hybridized with ^{35}P-dCTP-labeled cDNA probes generated by random-primed synthesis and washed at high stringency (2 × SSC, 65°C). The blot was then exposed to autoradiography film overnight. Total RNA from nontransfected cells and cells transfected with NF180 in the antisense orientation were run in the same gels as controls.

NF180 expression and purification in a prokaryotic system

The NF180 gene was cloned into a prokaryotic expression vector, pET-11b, and expressed in BL21 (DE3) pLysS

cells (Novagen). The cells were incubated overnight at 37°C in 1 L of LB containing 50 μg/ml of ampicillin in a shaking incubator at 225 rpm. Absorbance measurements were taken at 600 nm every 30 minutes until a reading of 0.8 was reached. Then expression was induced by 1 mM IPTG. An aliquot of cells was pelleted and suspended in 20 μl of SDS sample buffer and separated in 7.5% acrylamide SDS gel as described above. The gel was stained with Coomassie Blue to confirm expression of the 180 kD protein.

Cells were lysed and extracted by the method of Nagai and Thøgersen (1987). The cells from the 1-L culture were collected by centrifugation and suspended in 40 ml of lysis buffer containing 50 mM Tris-HCl, pH 8.0, 25% sucrose, and 1 mM EDTA. The suspension stood on ice for 30 minutes. Five ml of lysozyme (20 mg/ml) was added and the mixture was frozen at –20°C to assist in lysing the cells. Samples were thawed and 450 μg of DNase was added to digest DNA in the presence of 1 mM $MnCl_2$ and 10 mM $MgCl_2$ on ice for 30 minutes. The mixture was then extracted at 4°C for 10 minutes with the detergent solution that contained 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% deoxycholic acid, 1% Nonidet P-40, and 2 mM EDTA. The sample was then spun down and the supernatant was collected. In some experiments NF180 was found in the pellet (inclusion body) after detergent extraction. In that case the pellet was dissolved in column buffer and processed as described below.

Proteins were concentrated by the addition of 55% saturated ammonium sulfate. The sample was stirred for 1 hour at 4°C. The solution was centrifuged at 7,000*g* for 15 minutes. A small volume of column buffer containing 10 mM sodium phosphate, 6M urea, 1 mM dithiothreitol (DTT), and 0.4 mM phenylmethylsulfonyl fluoride (PMSF) at pH 7.5 was added to the loose pellet and mixed gently to dissolve the precipitate. The samples were placed in dialysis tubing and dialyzed against 1 L of column buffer overnight and then switched to a fresh 1 L of column buffer for another overnight dialysis. Anion exchange resin DE650W was balanced in 6M urea column buffer and packed in a 2.6 × 14 cm column. The samples were passed through the column at 80 ml/hr and eluted with 600 ml of salt gradient from 0–0.3M NaCl in 6M urea column buffer. Fractions were collected and analyzed on 7.5% acrylamide SDS gels as described above. The fractions containing the NF180 protein were pooled together and concentrated using a 2-ml column with the DEAE resin. The protein was eluted from the column using 12 ml of 6M urea column buffer containing 0.5M NaCl, during which 1 ml fractions were collected. The concentration of the protein in those fractions was measured using the Coomassie Plus Protein Assay (Pierce).

In vitro filament assembly

Purified NF180 (0.5 mg/ml in a volume of 50 μl) was placed in microcollodion tubes and dialyzed against a buffer system containing 4M GuHCl, 1 mM DTT, and 0.4 mM PMSF overnight at 4°C with stirring. The protein sample was then transferred into dialysis tubing and dialyzed for 3 hours at 37°C with stirring versus the assembly buffer containing 50 mM MES (morpholinoethanesulfonic acid), pH 6.25, 0.175M NaCl, 0.5 mM EGTA, 1 mM DTT, and 0.4 mM PMSF. In an attempt to identify optimal assembly conditions, NF180 self-assembly was also tested at other pH values (6.5 and 7.0), another buffer system (50 mM PIPES), a higher salt concentration (0.25M NaCl), and at lower temperatures (4°C, 15°C). Heterogeneous assembly was also tested in which the mammalian NF proteins NF-L, NF-M, NF-H, or vimentin were incubated with equimolar NF180 at optimum assembly conditions. Aliquots of the assembly reaction (10 μl) were applied to Formvar-coated copper grids and treated with 2% uranyl acetate. The copper grid was then allowed to dry for 5 minutes and checked under transmission electron microscopy (Jeol 1200EX-II).

BS^3 cross-linking and native gels

In order to determine whether NF180 forms homo-oligomers, protein samples were dialyzed in microcollodion tubes against a solution containing 20 mM TEA, 0.5 mM EDTA, 1 mM DTT, 0.4 mM PMSF, pH 7.8, overnight at 4°C with stirring. Four reactions were run with 5 μg of protein in each with increasing concentrations of the bis(sulfosuccinimidyl) suberate (BS^3) at 0M, 0.1 mM, 0.25 mM, and 0.5 mM. The reaction was allowed to proceed for 30 minutes at room temperature, then quenched with 5 μl of 50 mM Tris at pH 7.8. Then the samples were run on a 3% polyacrylamide and 0.5% agarose composite gel (Peacock and Dingman, 1968). NF-M was used as a control.

In order to study the possibility of NF180 forming a complex (hetero-oligomer) with the mammalian NF triplet proteins, 5% native gel was run as described in Cohlberg et al. (1995). Fifteen μg of each protein was used when run individually and also in combinations.

Photomicrography

Most images were captured by analog camera (Zeiss MC 100), developed on glossy photo paper, then scanned with an HP ScanJet 5300C. Images in Figure 8 were acquired with a confocal microscope (Leica TCS NT). PhotoShop software (v. 6.0, Adobe Systems, San Jose, CA) was used only to adjust the contrast and brightness of the images and to add labels.

RESULTS

Appearance of NF180 expressed in NIH3T3 cells

Transfected cells were heavily stained by NF180-specific antibodies (Fig. 1A). The expressed protein was evenly distributed in the cytoplasm of the cell bodies and their processes. This protein was indistinguishable in size from NF180 found in lamprey CNS extracts (Fig. 1B). By in situ hybridization, NF180 message was found mostly in the perinuclear cytoplasm (Fig. 1C). Northern blotting revealed a band of appropriate molecular weight (Fig. 1D). The NF180 protein expressed in NIH3T3 cells was heavily immunostained by mAbs directed at phosphorylation-independent and dephosphorylated epitopes of either the core or sidearm of NF180, but not phosphorylation-dependent epitopes (Fig. 2). Western blotting showed corresponding results (data not shown).

Shapes of NF180-positive and -negative cells are similar

This experiment was performed initially to test our hypothesis that overexpression of NFs would result in a protrusive force that contributes to elongation of cell processes. Therefore, either transiently or stably transfected

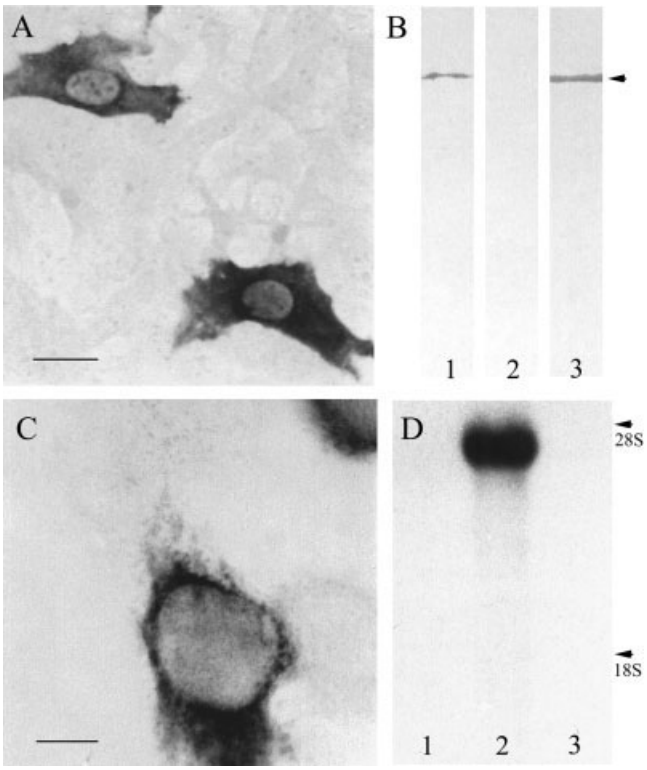


Fig. 1. NF180 expressed in NIH3T3 cells is the same size as the native protein. **A:** NIH3T3 cells were grown in DMEM medium and transfected with NF180 vector when they reached 80% confluence. Cells were immunologically labeled with NF180 antibody (LCM3), a phosphate-independent mAb binding to the core of NF180, and visualized with HRP-conjugated secondary antibody. **B:** Cells were homogenized and the whole proteins in the homogenate were resolved in SDS-gel. The proteins were transferred to nitrocellulose membrane and probed with another antibody LCM 39, which recognizes the carboxyl terminus sidearm of NF180. From left: transfected cells (lane 1), nontransfected cells (lane 2), and lamprey CNS (lane 3). **C:** In situ hybridization of NIH3T3 cells expressing NF180. The cells were transfected with NF180-expressing vector and probed in situ with digoxigenin-labeled cRNA derived from full length NF180 cDNA. **D:** Northern blot showing NF180 mRNA of correct size (3.7 kb); lane 1: transfected with NF180 in antisense orientation; lane 2: transfected with NF180; lane 3: cells without transfection. Positions of 3T3 cell's 28S and 18S ribosomal RNA are as indicated. Scale bar = 20 μm for A and 10 μm for C.

NIH3T3 cells were immunostained for NF180 and tubulin and the morphology of NF180-positive cells compared with that of NF180-negative cells (Fig. 3). The number of processes per cell (longer than cell nuclear diameter) and lengths of the processes (from the center of the cell nucleus to the end of the process) were quantified. There were no statistically significant differences between NF180-positive and -negative cells (Fig. 4).

Assembly properties of NF180 in cultured cell lines

Immunofluorescence of NIH3T3 cells expressing NF180 showed three different staining patterns with anti-NF180 mAbs: 1) filamentous arrays (Fig. 5A); 2) nonfilamentous aggregates (Fig. 5B); and 3) atypical fibrillar networks (Fig. 5C). The frequency of distributions for these three staining patterns can be appreciated in the low magnification image of Figure 5D, which shows that most NF180-positive staining appeared as atypical fibrillar networks.

Since NIH3T3 cells are derived from mouse fibroblasts, which possess a preexisting vimentin IF network, it was not clear whether filamentous structures represented NF180 self-assembly, coassembly of NF180 with endogenous vimentin, or decoration of a preexisting IF network. In order to distinguish these possibilities, NF180 was transfected into the human adrenal carcinoma cell line SW13cl.2vim$^-$ or SW13cl.1vim$^+$, which lacks or contains its own cytoplasmic IF network, respectively. The vast majority of SW13cl.2vim$^-$ cells transiently transfected with NF180 displayed nonfilamentous aggregates. In some cases they exhibited perinuclear clustering with NF180 staining (Fig. 6A). A small percentage of cells showed an atypical fibrillar network after NF180 staining, which may be due to contamination of the cultures with vimentin-positive cells, as reported previously (Lee et al., 1993). However, the majority of SW13cl.2vim$^-$ cells were not labeled by anti-vimentin antibodies (Fig. 6B). In NF180-transfected SW13c1.1vim$^+$ cells the staining pattern with anti-NF180 antibodies was predominantly an atypical fibrillar network (Fig. 6C). Staining with anti-vimentin antibodies showed a typical filamentous array (Fig. 6D). A small number of SW13c1.1vim$^+$ cells showed a fine, punctuate cytoplasmic staining pattern, similar to that of most vim$^-$ cells. The different distribution and morphology of NF180 in vim+ versus vim-SW13cl cells suggests a limited degree of interaction between vimentin and NF180, even though they do not assemble together to form long intermediate filaments. The same results were obtained when cultures were kept at 15°C or 25°C.

Coexpression of NF180 with mammalian NFs

NF180 was coexpressed in SW13cl.2vim$^-$ cells with each of three rat NFs (NF-L, NF-M and NF-H). Transient transfection of vectors expressing NF180 and NF-L resulted in the formation of atypical filamentous networks detectable with antibodies against NF-L or NF180 (Fig. 7A,B). Long, rod-like filamentous structures suggestive of densely bundled NFs were observed in all the NF-L/NF180 double-transfected cells. These rod-like structures were detectable with either NF-L or NF180 antibodies, suggesting coassembly between NF-L and NF180. However, cells coexpressing NF180 and NF-M (Fig. 7C,D) or NF-H (data not shown) showed nonfilamentous aggregates by NF180 or NF-M/NF-H immunostaining. No rod-like filamentous structures were observed.

To confirm that the recombinant NF180 retains the ability to be incorporated into true intermediate filaments, triple transfections of SW13c1.2vim$^-$ cells with NF-L, NF-M, and NF180 were performed. Expression of NF-M was confirmed in most transfected cells with an anti-NF-M antibody. Then double-labeling was performed with antibody LCM3 for NF180 and an anti-NF-L antibody (Sigma). The triply transfected cells now showed typical filamentous structures that were both NF-L and NF180-positive (not shown). Since NF180 and NF-M have significant sequence similarities, it is necessary to rule out cross-reaction of LCM3 with NF-M as a possible cause of false-positive identification of NF180 incorporation into NFs. Therefore, NF180 was tagged with GFP and detected with anti-GFP antibody. In con-

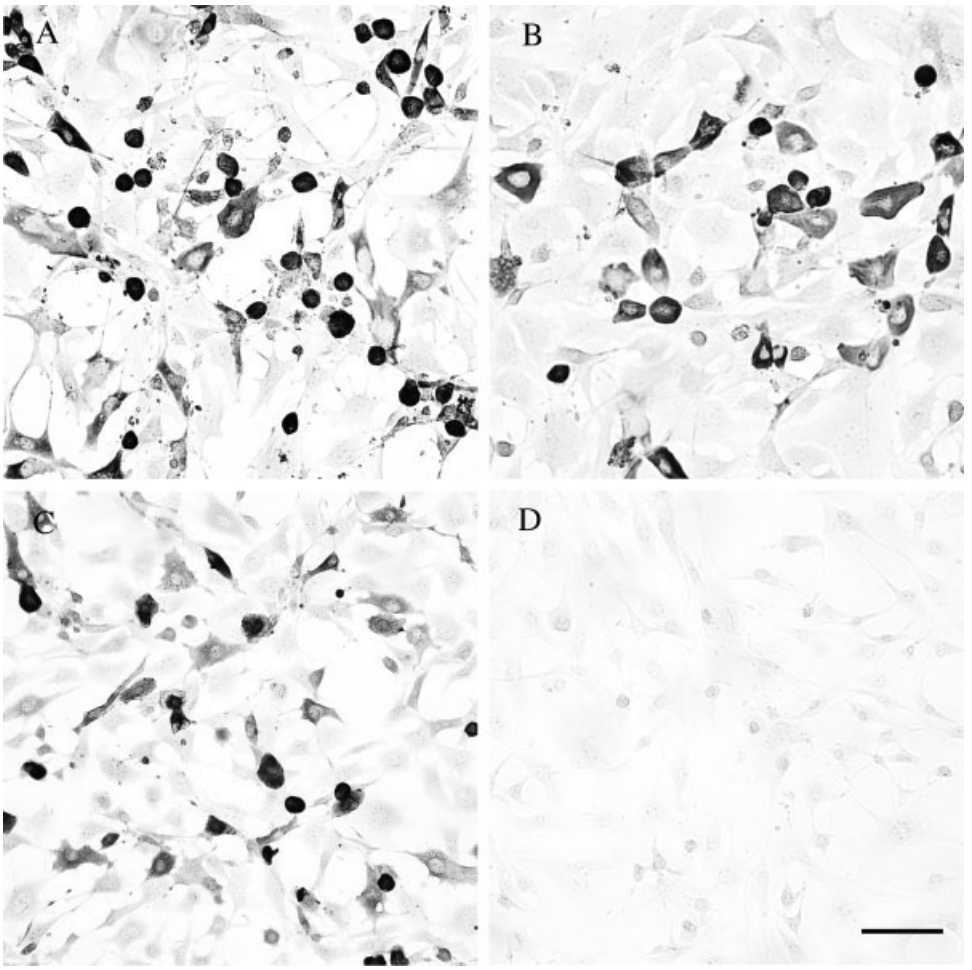


Fig. 2. NF180 protein expressed in transiently transfected NIH3T3 cells was poorly phosphorylated. NIH3T3 cells were transfected with the NF180 expression vector at 80% confluence and immunostained with four individual antibodies. **A:** LCM3, a phosphate-independent antibody binding to the core of lamprey NF180. **B:** LCM39; a phosphate-independent antibody binding to the sidearm of lamprey NF180. **C:** RMdO15, binding to the non-phosphorylated isotype of the sidearm. **D:** RMO62, binding to phosphorylated isotypes of the sidearm. Scale bar = 40 μm.

trol experiments, this antibody did not label any cells that were transfected with NF-L or NF-M, suggesting there is no cross-reaction between anti-GFP mAb and NF-L or NF-M. However, when SW13cl.2vim$^-$ cells were triple-transfected with NF-L, NF-M, and GFP-NF180, double-labeling for GFP-NF180 (Fig. 8A) and NF-L (Fig. 8B) revealed typical filamentous structures that contained both NF-L and recombinant NF180 (Fig. 8C). This experiment also shed light on the stoichiometry between NF180 and NF-L and on the nature of the nonfilamentous aggregates seen in cells transfected with NF180 alone. The three cells in Figure 8 all show similar staining intensity for NF180 (Fig. 8A), but the intensity of NF-L staining varies. Thus, the cell on the left appears to express the highest levels of NF-L, while the cell in the lower right contains the lowest levels of NF-L (Fig. 8B). When NF-L levels are high, as in the two left-most cells, all the NF180 was incorporated into the typical filamentous network. However, when NF-L levels were low, as in the right-most cell, some of the NF180 was found in nonfilamentous aggregates. The different ratios of NF-L to NF180 contents were reflected in the different tints of the double-labeled image (Fig. 8C). Thus, the recombinant NF180 is capable of incorporation into NFs if it is combined with other appropriate subunits.

Failure of NF180 self-assembly is not temperature-dependent

Since the lamprey is a cold-blooded vertebrate, the ability of NF180 to self-assemble might be dependent on temperature or the species from which the transfected cells were derived. Three fish cell lines, RTG-2, CHES-214, and RTH-149, were tested. However, no evidence was observed to support the formation of typical filamentous arrays of NF180 at an incubation temperature of 18°C. The NF180 formed either nonfilamentous aggregates (Fig. 9A) or atypical, fine fibrillar networks suggestive of very short filaments (Fig. 9B) similar to those seen in NIH3T3 cells (Fig. 5).

In vitro assembly of NF180

NF180 expressed in bacteria was purified and its identity confirmed by Western blot (Fig. 10A) using two mAb's, LCM39 (left panel), and LCM3 (right panel). LCM39 recognizes an epitope on the carboxy terminus sidearm of NF180, while LCM3 recognizes an epitope in the core region (Jacobs et al., 1995). Since other intermediate filaments, such as keratin or vimentin, share conservative core regions with neurofilaments, LCM3 also binds to multiple bands around 50 kDa that may be the keratin or other intermediate filaments (Fig. 10A,

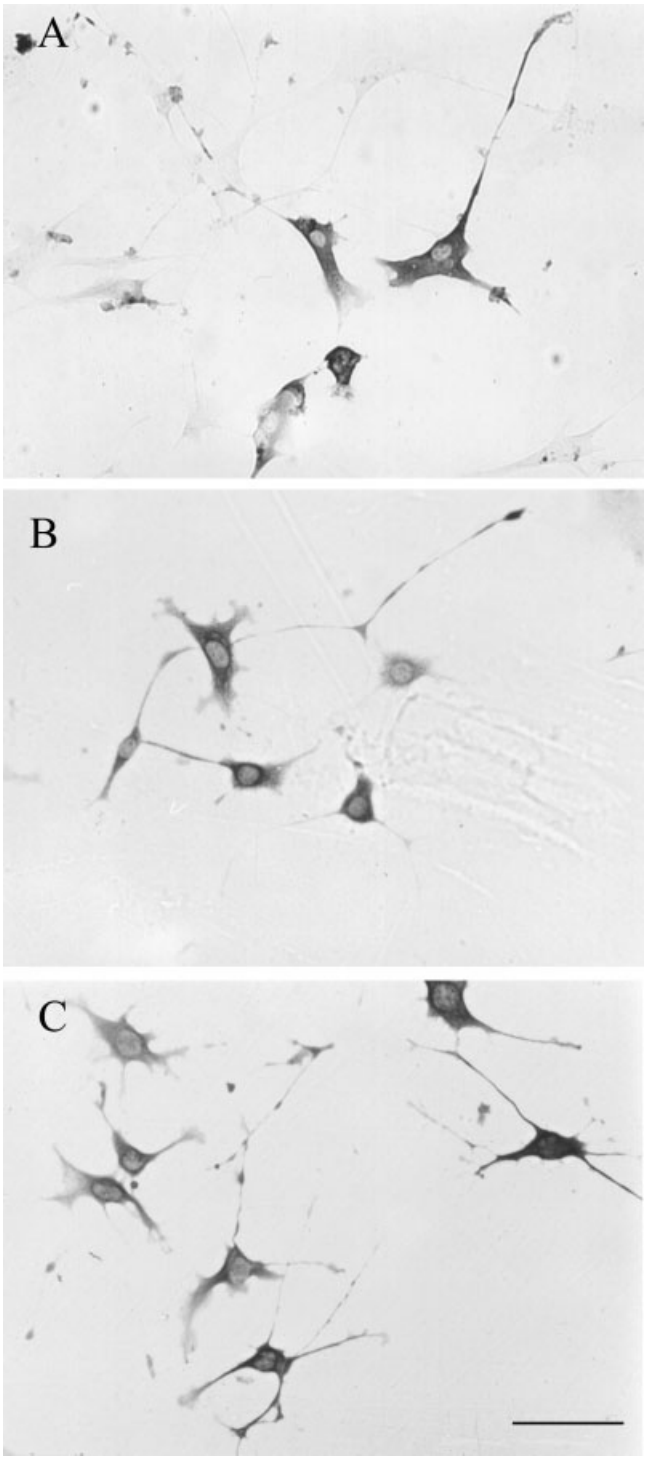


Fig. 3. Expression of NF180 does not alter the shapes of NIH3T3 cells. Transient (**A**) and stably transfected (**B**) NIH3T3 cells stained by an antibody to NF180 (LCM3). **C:** Control cells stained by an antibody to total α-tubulin (DM1A, Sigma). Scale bar = 40 μm.

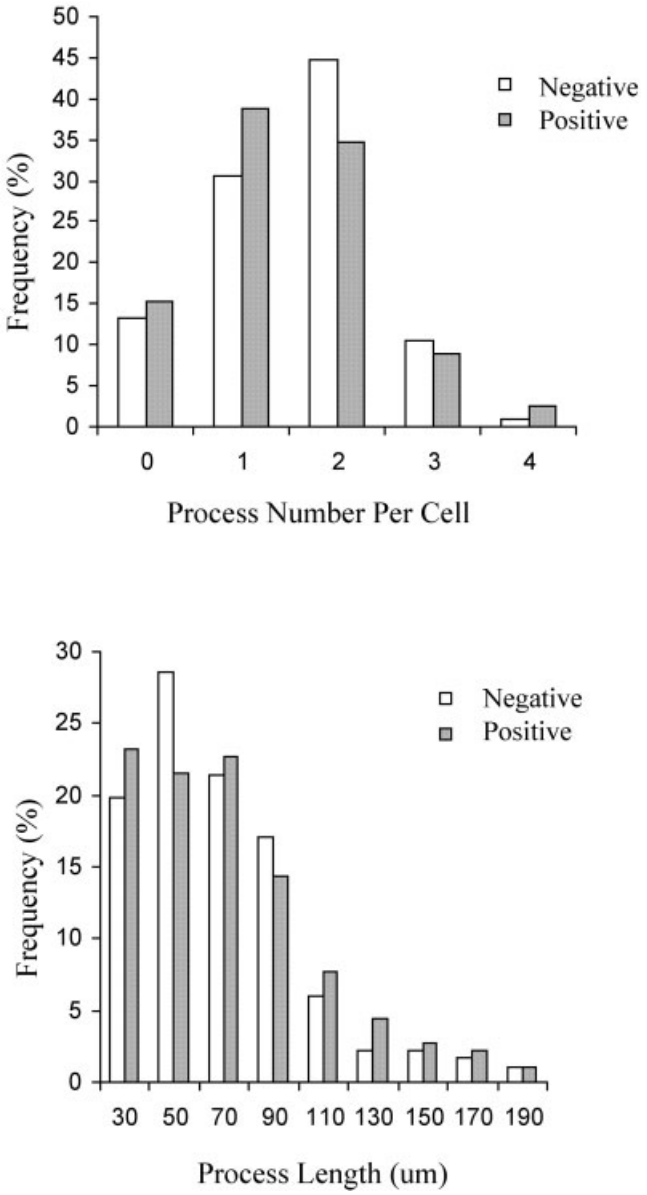


Fig. 4. Expression of NF180 does not alter the lengths or numbers of NIH3T3 cell processes. Control cells and cells expressing NF180 were quantitated for the number of processes per cell and the length of each process. The number of processes per cell for transfected cell was 1.44 ± 0.93 (n = 124), which was also not different from control cells (1.55 ± 0.88, n = 115). The mean length of processes for NF180 positive cells was 71.5 μm ± 36.9 SD (n = 181), not significantly different from control cells (67.4 μm ± 34.8, n = 182).

lane 7), and to rat NF-M (Fig. 10A, lane 8). By contrast, LCM39 is highly specific for NF180. In lanes 1 and 5 of Figure 10A, multiple lower molecular weight bands just below the predominant NF180 band probably represent minor protein degradations. A series of self-assembly experiments was performed under different conditions (pH and salt concentration) in vitro. At pH 6.25, 37°C, and 0.175M NaCl, NF180 formed no filamentous structures. Rather, it appeared as short rods and fragments on EM (Fig. 10B). When these in vitro self-assembly experiments were repeated at 15°C or 4°C or at higher salt concentration (0.25M NaCl), atypical fibrillar networks were seen on EM only at 15°C (Fig. 10C). These

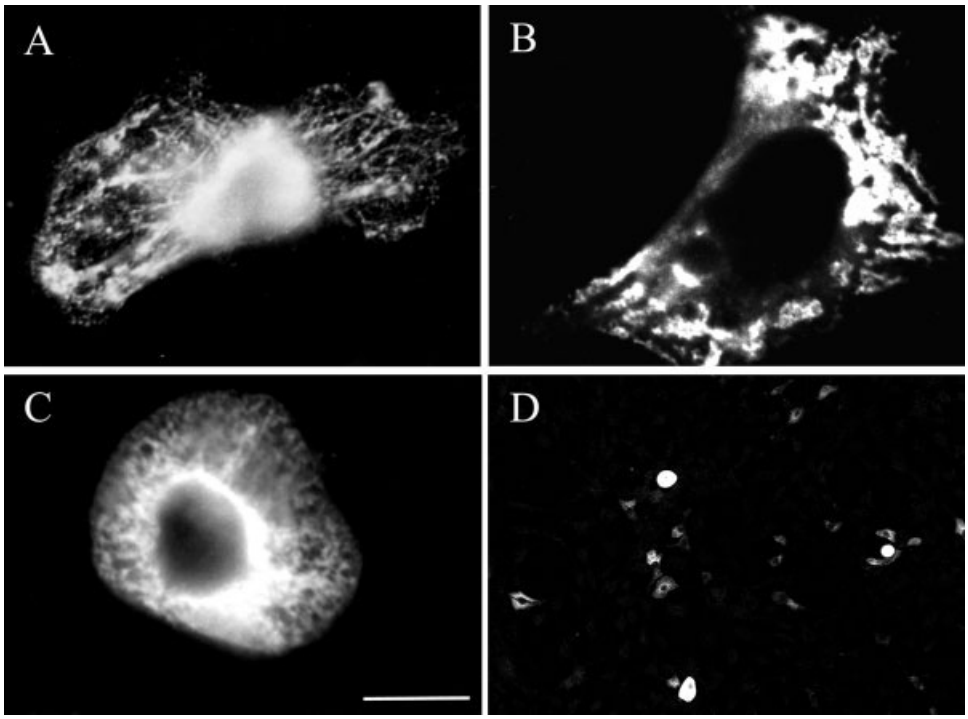


Fig. 5. Structural organization of NF180 expressed in NIH3T3 cells. NF180 assumed one of three different appearances when it was transiently transfected into 3T3 cells. **A:** Filamentous arrays. **B:** Nonfilamentous aggregates. **C:** Atypical fibrillar networks evenly distributed in the cells. **D:** Lower magnification image of NF180 expressed in NIH3T3 cells. Scale bar = 10 μm in C (applies to A–C), 100 μm for D.

atypical networks differed markedly from the filaments generally seen with NF-L self-assembly (Fig. 10D). Thus, the results of in vitro tests were consistent with the observations obtained in cultured cells and suggested that NF180 cannot self-assemble into long filaments.

NF180 was also tested for coassembly with equimolar concentrations of NF-L, NF-M, and NF-H at pH 6.25 be-

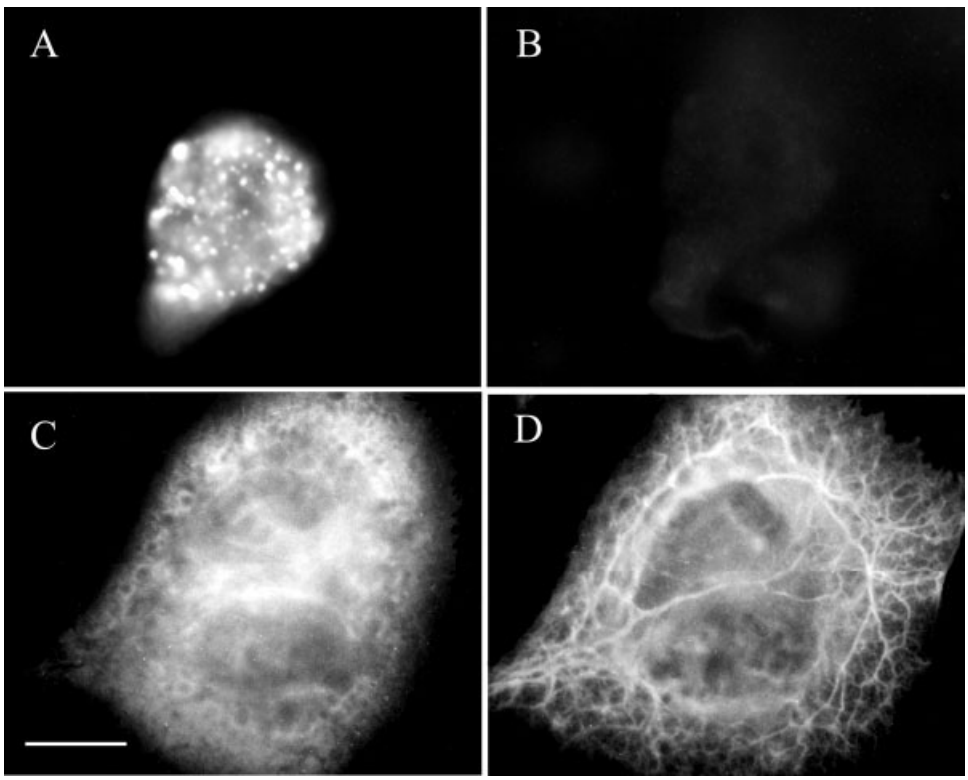


Fig. 6. Structural organization of NF180 expressed in SW13c1.1/SW13c1.2 cells. Both SW13c1.2vim$^-$ cells (top row) and SW13c1.1vim$^+$ cells (bottom row) were transfected with NF180 and stained with an antibody against NF180 (left column) or vimentin (right column) at 37°C. No filamentous structures were found in either cell line. These frames show that NF180 was not colocalized with the vimentin filamentous network. Scale bar =10 μm.

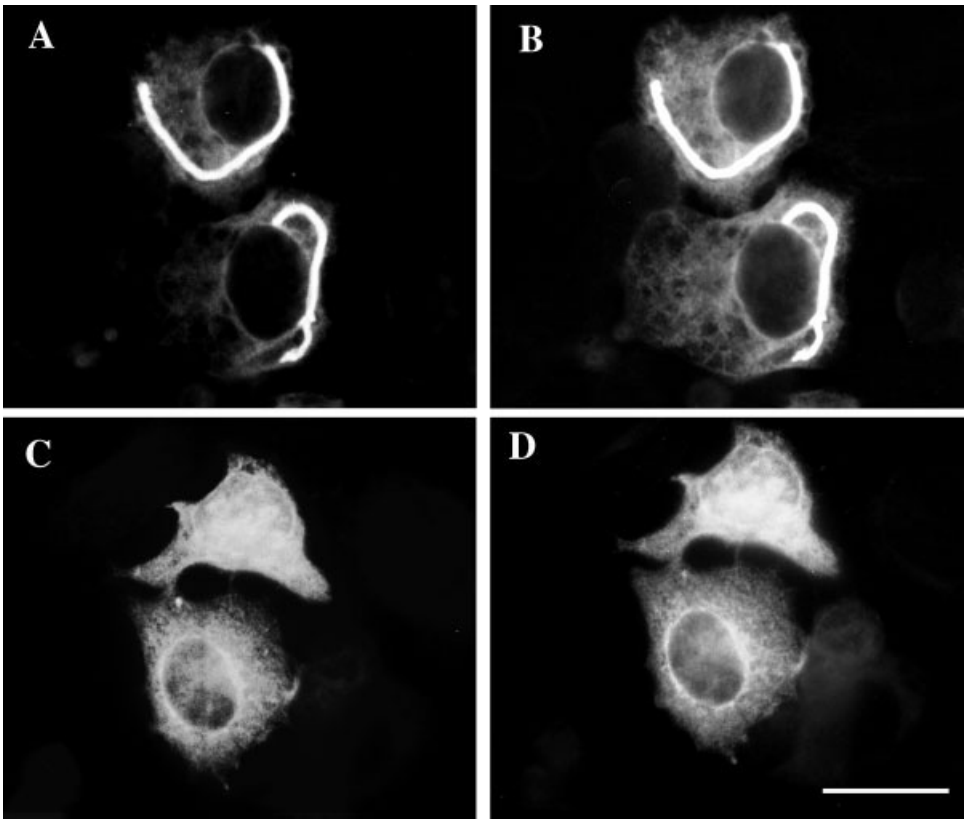


Fig. 7. Structural organization of NF180 coexpressed in SW13cl.2vim$^-$ cells with mammalian NF triplet proteins. NF180 was transfected into SW13cl.2vim$^-$ cells that were also transfected with one of the rat NF triplet proteins and double-labeled with LCM3 mAb specific for NF180 and the polyclonal antibodies for NF-L or NF-M (Beaulieu et al., 1999). **A:** Cells cotransfected with NF180 and NF-L labeled for NF-L. **B:** The same cells imaged for NF180. Note the colocalization of NF-L and NF180 in dense rod-like neurofilamentous arrays. **C,D:** Cells were cotransfected with NF180 and NF-M and imaged for NF-M and NF180, respectively. Only nonfilamentous aggregates were seen. The suggestion of partial colocalization is probably due to cross-reactivity between the anti-NF180 antibody and NF-M (see Fig. 10A). Scale bar = 20 μm.

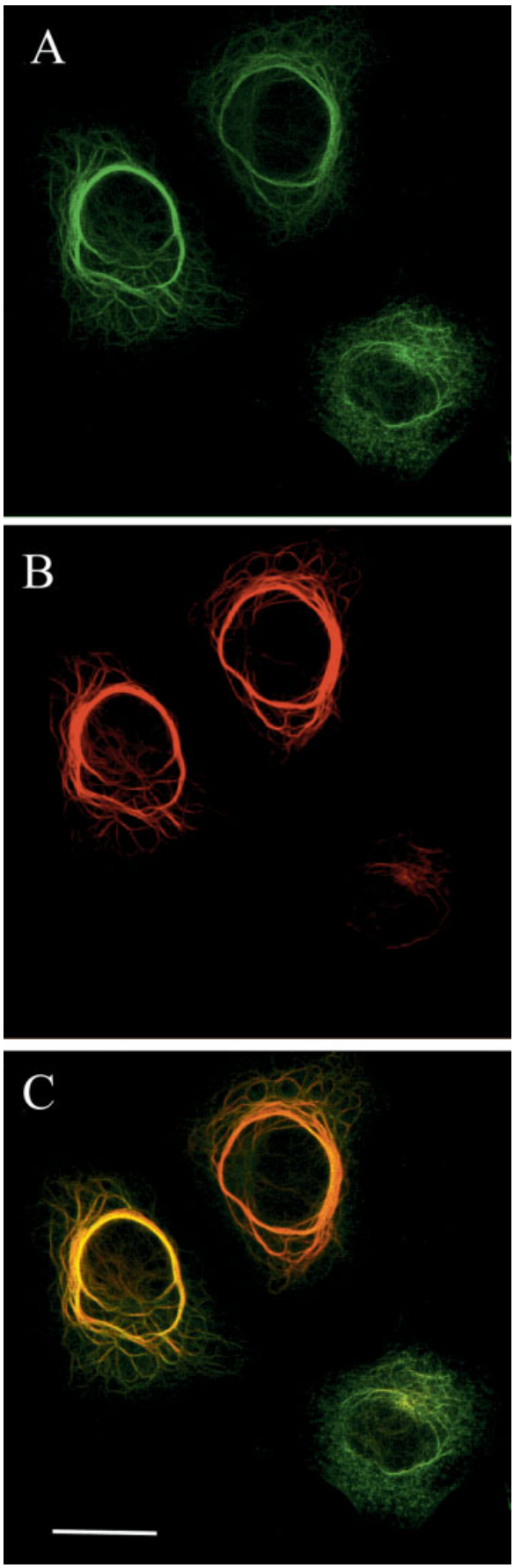
cause mammalian NF-L formed typical filamentous structures at this pH, or with vimentin at pH 7.25 because of its preference for higher pH. NF180 did not form filamentous structures with any of these mammalian IF proteins. Only short fragments and nonfilamentous aggregates were observed in the NF180 and NF-L coassembly test. Furthermore, NF180 appeared to disrupt the self-polymerization that normally occurs with NF-L, suggesting that NF180 binds to some epitopes in NF-L that are necessary for self-assembly (data not shown).

BS^3 cross-linking experiments were performed to determine whether NF180 forms homo-oligomers. Rat NF-M was selected as a control because it has a molecular weight similar to that of NF180. In the absence of BS^3, NF180 formed a single band on 3% polyacrylamide 0.5% agarose gel electrophoresis (Fig. 11A). Addition of up to 0.5 mM BS^3 did not result in the appearance of a second band, nor in the loss of intensity of the monomeric band, suggesting that even in the presence of a cross-linking reagent, NF180 does not form homodimers. By contrast, NF-M showed a faint second band of higher molecular weight and a slight loss of intensity of the monomeric band with increasing concentrations of BS^3, indicating that NF-M does form homodimers to a limited extent (Fig. 11A). In parallel experiments analyzed on 6% gels, NF-L showed extensive formation of dimers and tetramers when treated with BS^3 (not shown).

Nondenaturing polyacrylamide gel electrophoresis was used to determine whether NF180 could form a complex (hetero-oligomer) with mammalian NF triplet proteins (Cohlberg et al., 1995). Native gels separate by both size and charge. A 5% urea gel shown in Figure 11B demonstrates the electrophoretic patterns of 15 μg of individual proteins NF-L, NF-M, NF-H, and NF180, and the combination of NF180 with NF-L, NF-M, and NF-H, respectively. NF-L migrates as a homodimer in this gel system (Athlan and Mushynski, 1997). NF-L showed a second, albeit faint, higher molecular weight band, which may represent homotetramers. Combination of NF-L with NF180 resulted in the disappearance of the NF180 band and an increase in the intensity of the upper band (indicated by star and arrow, respectively), suggesting the formation of heterodimers between NF-L and NF180. When NF180 was mixed with NF-M or NF-H, there was no change in the intensity of the NF180 band and no new bands were formed (Fig. 11B), suggesting that NF180 did not form complexes with NF-M or NF-H. The results of the native gel experiments are consistent with the findings of the coassembly experiments in cell culture and in vitro, i.e., NF180 appeared unable to form complexes with the NF-M or NF-H, but did form complexes with NF-L.

DISCUSSION

Previous studies of lamprey cytoskeletal proteins failed to find evidence for more than one NF subunit (Pleasure et al., 1989; Jacobs et al., 1995). It was therefore assumed that NF180 self-assembles into NFs, thereby providing a convenient model for testing the role of NF in axonal regeneration. The present results indicate that NF180 lacks the ability to self-assemble and that expression of NF180 in NIH3T3 cells does not significantly affect the



length or number of their processes. That the failure of NF180 to self-assemble is not an artifact of the specific techniques and cell lines employed is suggested by the ability of NF180 to form thick filamentous bundles with rat NF-L and to be incorporated into typical filamentous networks in the presence of NF-L and NF-M (see below).

NF180 cannot self-assemble

NF180 failed to self-assemble when expressed in NIH3T3 or SW13c1 cell lines. The reason for the three different expression patterns of NF180 in NIH3T3 cells is not clear, but it seems plausible from comparisons between expression patterns in vimentin-positive and vimentin-negative cells that the patterns resulted from varying levels of expression. When NF180 expression is low, it is distributed as small particles in the cells and may sparsely decorate some cytoskeletal proteins, which gives rise to an appearance of a filamentous array (Fig. 5A). As the level of expression increases, NF180 may densely decorate the cytoskeleton, leading to the appearance of an atypical fibrillar network (Fig. 5C). Further increase in the expression of NF180 would have resulted in their denser accumulation as nonfilamentous aggregates (Fig. 5B). Since both of these cell lines are of mammalian origin, they might lack the ability to assemble IFs of nonmammalian species, especially poikilotherms. For example, trout vimentin failed to form IF arrays when transfected into vimentin-free mammalian cells cultured at 37°C (Herrmann et al., 1996). Similar observations were reported for *Xenopus* vimentin (Herrmann et al., 1993). Reduction of the incubating temperature to 28°C permitted *Xenopus* vimentin to reorganize into normal IF arrays. In order to evaluate this possibility, NF180 was transfected into fish cell lines, including RTG-2, CHES-214, and RTH-149, which were cultured at temperatures of 18°C. However, only atypical fibrillar networks, but not typical IF arrays, were observed in fish cell cultures under reduced temperatures.

The atypical fibrillar networks observed in these cells may represent the formation of very short heteropolymers that were not integrated into the main filamentous structure. This is consistent with the EM appearance of NF180 assembled in vitro at 15°C, which suggested the possible formation of higher-order protofilaments, but not formation of long filaments. However, even in the presence of the cross-linking compound BS^3, NF180 could not form covalent homodimers in vitro, as did NF-L and, to a lesser degree, NF-M. The incompetence of NF180 to form homo-oligomers in BS^3 is consistent with its inability to self-polymerize in vitro and in cultured cells. But in view of this, it is not yet clear what the short structures observed on EM images of NF180 in vitro represent.

The self-assembly of NF180 seemed to be supported by a recent study in which EYFP-fused NF180 transfected into lamprey brainstem neurons induced perikaryal swelling, enlargement of axon diameters, and increases in the

Fig. 8. NF180 forms a typical filamentous network with NF-L and NF-M in SW13cl.2vim$^-$ cells. GFP-tagged NF180 was cotransfected with NF-L and NF-M into SW13cl vimentin$^-$ cells. The cells were kept in incubator at 37°C for 48 hours and double-labeled with an anti-GFP antibody for NF180 (**A**) and an anti-NF-L antibody (**B**). Merged image (**C**) corresponding to staining with both antibodies. NF180 was incorporated into a typical filamentous network with NF-L and NF-M. Scale bar = 20 μm.

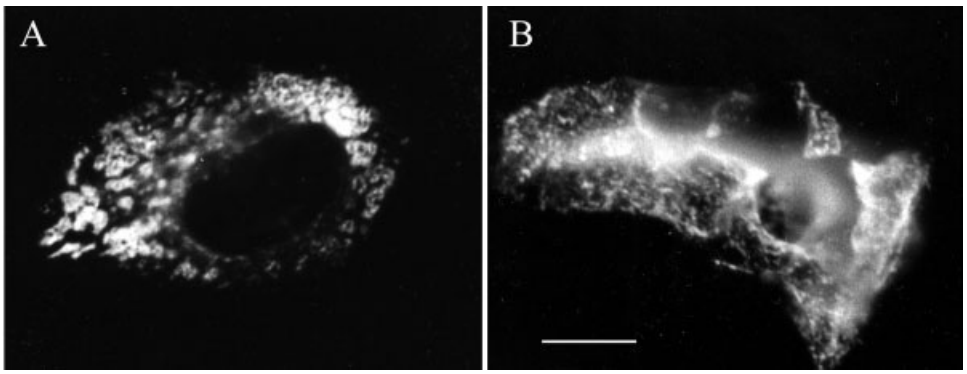


Fig. 9. NF180 expression in a fish cell line. CHES-214 cells, derived from Chinook salmon embryos, were transfected with NF180 and stained with an NF180-specific mAb. Two staining patterns were observed: (**A**) aggregates and (**B**) atypical fibrillary networks suggestive of incompletely assembled NF. Scale bar = 10 μm.

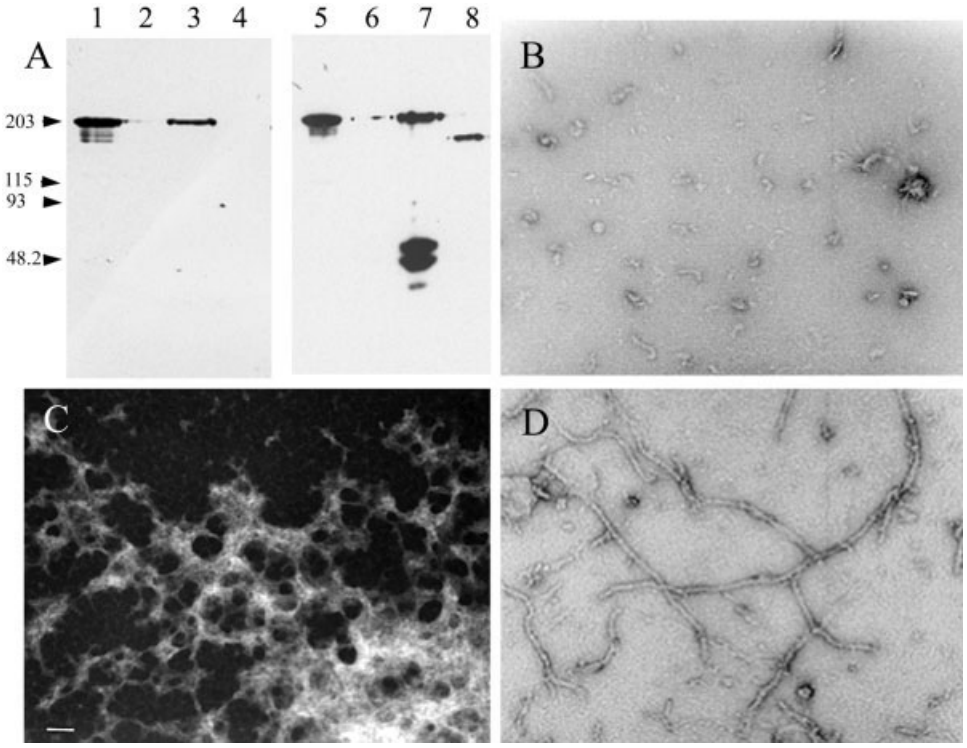


Fig. 10. NF180 does not self-assemble in vitro. NF180 was expressed in *E. coli* and its reconstitution in vitro studied by Western blot and EM. **A:** Western blot of bacterial homogenates, showing that the recombinant NF180 is recognized by two anti-NF180 antibodies (LCM39 for left panel, and LCM3 for right panel) and has a size identical to that of native NF180 extracted from lamprey CNS. Lanes 1 and 5 are samples obtained from bacteria; lane 2 and 6 are samples obtained from lamprey brain; lanes 3 and 7 are samples from lamprey spinal cord; and lanes 4 and 8 are the samples from rat spinal cord. **B:** EM of NF180 reconstituted in 0.175 M NaCl at pH 6.25, showing clumps of NF180. **C:** EM of NF180 reconstituted at pH 6.5 and 15°C showing a pattern similar to that predicted by atypical, fine fibrillary networks. **D:** EM of rat NF-L self-assembly under the same conditions as in B. Scale bar = 100 nm.

numbers of IFs (Hall et al., 2000). However, this study did not present evidence that the recombinant NF180 was incorporated into NFs as opposed to inducing the overproduction of native NFs. If the trans-NF180 was incorporated into NFs, it might have coassembled with other endogenous IF proteins. EYFP-NF180 transfected into NB2a (neuroblastoma) cells also induced an increase in cell diameter, but it could not be determined from the immuno-EM images whether the transfected NF180 self-assembled, coassembled with mammalian NF subunits, or merely decorated some preexisting NFs. Immunofluorescence images suggested that the NF180 did not form filaments.

NF180 cannot form filamentous networks with vimentin

Vimentin is a homopolymeric type III IF. It can self-assemble and coassemble with each of the mammalian NF triplet subunits (Monteiro and Cleveland, 1989; Chin and Liem, 1990). However, NF180 was incapable of forming filamentous arrays with vimentin. We expressed NF180 in 3T3 cells and found that most cells had atypical fibrillar networks on NF180 immunostaining. Since this cell line is derived from fibroblasts, which possess a vimentin fibrous network, SW13c1.1vim$^+$ and SW13c1.2vim$^-$ cells were transfected with NF180 vector and immunostained for NF180 or vimentin. In these cells, double-labeling for vimentin and NF180 showed that NF180 neither colocalized with the vimentin filamentous network nor disrupted it. There was a difference in appearance between the non-filamentous aggregates of NF180 labeling in vim$^-$ cells and the atypical fibrillary arrays seen in vim$^+$ cells. The reason for this is not known, although it is possible that the fibrillary arrays represent the formation of incomplete filaments. Thus, NF180 neither coassembles with mammalian vimentin to form long filaments nor decorates long

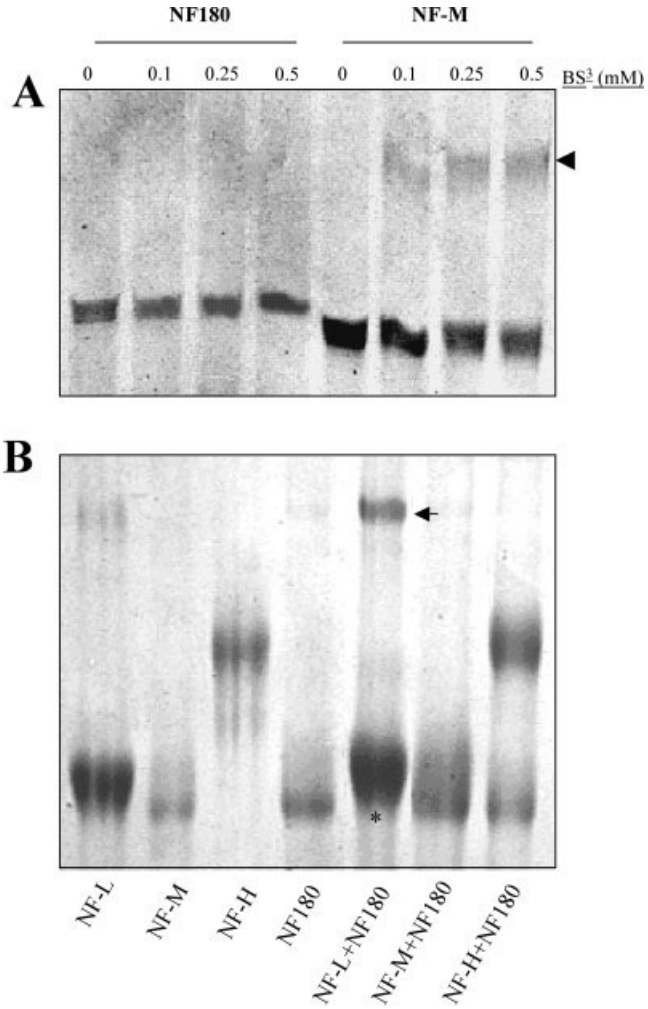


Fig. 11. NF180 does not form homodimers or heterodimers with mammalian NF-M or NF-H, but does form a heterodimer with mammalian NF-L. **A:** NF180 was cross-linked with BS^3 and analyzed by 3% polyacrylamide and 0.5% agarose composite gel. Note that no additional bands appear and the NF180 band does not become fainter as BS^3 concentration is increased, indicating that no dimers or oligomers are formed. As a control, the same cross-linking reactions were performed with NF-M. Note that as the concentration of BS^3 increases, a second band of higher molecular weight appears (arrowhead) and the NF-M monomeric band becomes lighter, indicating that NF-M dimers have formed. **B:** 5% native gel shows that NF180 had interaction with NF-L, but not with NF-M and NF-H. Note in lane 5 the loss of the NF180 band (*) and the appearance of a third band of higher molecular weight (arrow), suggesting the formation of an NF-L-NF180 complex. This is not apparent with NF-M or NF-H in lanes 6 and 7.

vimentin filaments. However, it is not known whether NF180 can coassemble with lamprey vimentin.

NF180 forms filaments with NF-L, or NF-L plus NF-M, but not with NF-M or NF-H

Among the vertebrate NF subunits, NF180 shows the greatest sequence homology with human NF-M (Jacobs et al., 1995). NF-M is able to form heteropolymeric filaments with NF-L in vivo (Ching and Liem, 1993; Lee et al., 1993) and in vitro (Hisanaga and Hirokawa, 1990). This raised the possibility that NF180 could form heteropolymeric filaments if mixed with the NF triplet proteins. NF180 did form rod-like, dense filamentous structures in SW13cl.2vim$^-$ cells cotransfected with NF-L, but not with NF-M or NF-H. Although these filamentous structures differ from typical filamentous networks, they suggest a close interaction between NF180 and NF-L. Moreover, when NF180 was cotransfected with NF-L plus NF-M, it formed typical filamentous structures in the same cell line (Fig. 8). There exists a stoichiometry between NF180 and NF-L in the presence of NF-M, such that the excess expression of NF180 over NF-L formed nonfilamentous aggregates. Similarly, when NF180 was mixed with equimolar concentrations of NF triplet proteins in vitro and dialyzed in assembly buffer, it formed heterodimers and/or oligomers with NF-L but not with NF-M or NF-H. The reason for the permissive effects of NF-M on the formation of typical filamentous structure by NF180 and NF-L is not known. It is possible that a longer tail of NF-M provides more position, from which a bifurcation is originated. What this added effect of NF-M means for the mechanism of NF assembly in the lamprey is also not known. However, the frog is reported to have two isoforms of NF-M (Gervasi and Szaro, 1997). Perhaps NF180 is similarly heterogeneous and assembly might be facilitated by the mixture of more than one NF180 isotype.

On the basis of correlative morphological and in situ hybridization data, this laboratory postulated a role for NFs in the mechanism of regeneration (Lurie et al., 1994; Hall et al., 1997; Jacobs et al., 1997). The densely packed NFs in the tips of regenerating axons (Lurie et al., 1994) and the paucity of F-actin in ectopically regenerating lamprey growth cones (Hall et al., 1997) suggested that synthesis and transport of NFs into the growing tip might impart stiffness (Pijak et al., 1996) and generate a protrusive force that contributes to axonal regeneration. If this is true, overexpression of NF180 might result in accelerated regeneration. We originally undertook the experiments on NIH3T3 cells as a simple way of testing this hypothesis, with the expectation that enhanced production of NFs might result in faster cell process growth and longer cell processes. As indicated above, this was not the case. Although this might be because the neurofilament hypothesis of regeneration is not correct, other explanations are possible. NF180 might generate its protrusive force only when incorporated into filaments or only into highly phosphorylated filaments. Immunohistochemical studies in lamprey suggested that the NFs in the growth cones of regenerating axons are present in a highly phosphorylated form (Pijak et al., 1996; Hall et al., 1997), the form in which NFs normally exist in the largest caliber axons (Nixon et al., 1994; Pijak et al., 1996), and would be expected to generate the greatest degree of stiffness. However, the recombinant NF180 was present in transfected cells of the present experiment in poorly phosphorylated form. It may be that the protein kinases related to phosphorylation of NF180 are not expressed in these cell lines. Alternatively, the kinases may require activation by a specific signal that is absent in these cell lines. The microtubular network in NIH3T3 cells, which are derived from fibroblasts and not neurons, may not be suited to the optimal transport of NF180, so that the subunit could not generate a sufficient protrusive force to affect process elongation. Thus, it is possible that a neuronally derived cell line, such as neuroblastoma, would be more suitable.

In any case, the failure of NF180 to self-assemble in vitro and in cells that support the assembly of hybrid NFs from NF180 and NF-L suggests that, similar to mammalian NF-M and NF-H, NF180 may require another NF-L-like element for filament assembly.

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