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Assembly Properties of Lamprey Neurofilament Subunits and Their Expression After Spinal Cord Transection

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Abstract

In mammals neurofilaments (NF) are formed by coassembly of three subunits: NFL, NFM, and NFH (light, medium, and heavy). It had been believed that lampreys have only one subunit, NF180. However, a previous study showed that NF180 could not self-assemble but could coassemble with rat NFL, suggesting the existence of additional NF subunits in lamprey. More recently, we cloned three additional NF subunits. These new subunits and NF180 have now been transfected in combinations into SW13cl.2Vim⁻ cells, which lack endogenous cytoplasmic intermediate filaments. None of the subunits could self-assemble. No combination of NF subunits could form filaments in the absence of lamprey NFL (L-NFL). Assembly occurred at 28°C, but not at 37°C. L-NFL could form thick NF bundles with NF180 but not with NF132 and NF95, which formed only fine filamentous arrays. To determine which parts of the NF subunits are required for filament or bundle formation, we constructed deletion mutants of NF180 and cotransfected them with L-NFL. As with mammalian NF, only constructs with intact head and core domains could form filaments with L-NFL. However, the full length of NF180 was required to form NF bundles. As with NF180, in situ hybridization indicated that mRNA for L-NFL and NF132 was downregulated in identified reticulospinal neurons by 5 weeks after spinal cord transection, but was reexpressed at 10 weeks selectively in those neurons whose axons have a high probability of regenerating. This is consistent with a possible role of NFs in the mechanism of axon regeneration.

INDEXING TERMS

neurofilaments; coassembly; sea lamprey; transfection

Regenerating axon tips in transected sea lamprey (*Petromyzon marinus*) spinal cord contain dense accumulations of neurofilaments (NFs) (Lurie et al., 1994; Pijak et al., 1996; Hall et al., 1997) and NF expression by individual neurons after axotomy is correlated with their regenerative ability (Jacobs et al., 1997). This has suggested that NFs may play a role in the mechanism of axonal regeneration. NF proteins are intermediate filaments (IFs) found in the neuronal cytoskeleton. Mammalian NFs are composed of three polypeptide subunits: NFL, NFM, and NFH corresponding to light, medium, and heavy molecular mass. Rodent NFs are obligate heteropolymers, i.e., NFL must bind to either NFM or NFH to form filaments. By comparison, NFs in lampreys had been thought to contain only a single subunit, NF180

(Jacobs et al., 1995), which implied that NF180 self-assembles. However, in a previous study (Zhang et al., 2004) NF180 could not self-assemble in vitro, nor in cell cultures, including mammalian cell lines cultured at 37°C and fish cell lines cultured at lower temperatures. Furthermore, NF180 appeared to disrupt self-polymerization of rat NFL in vitro, and NF180 formed thick, rod-like filamentous structures when coexpressed with rat NFL in SW13c1.2Vim⁻ cells, which lack endogenous IFs. More typical filament networks were observed when NF180 was coexpressed with NFL plus NFM. Transfection of NF180 into NB2a/d1 neuroblastoma cells, which contain endogenous NFs (Shea et al., 1985) resulted in the incorporation of NF180 into NFs (Hall et al., 2000). Thus, NF180 may require an NFL-like protein and possibly additional elements to form normal filaments in lamprey. Indeed, a 50 kDa neuronal IF (nIF50) was described based on sodium dodecyl sulfate (SDS) gels and immunohistochemistry (Jin et al., 2005). In attempting to clone this missing nIF we recently cloned and sequenced three new NF genes (Jin et al., 2011), two of them NFM-like peptides (NF132 and NF95) and the third a lamprey NFL homolog (L-NFL). None of the new sequences represented nIF50, the sequence of which is still unknown.

In the present study the assembly properties of the four sequenced lamprey NFs and of truncated NF180 constructs were determined in SW13c1.2Vim⁻, a well-established system for assessing the assembly requirements of IFs, including NFs, due to its lack of any endogenous IF proteins (Sarria et al., 1994). The results suggest that L-NFL is essential to NF assembly and the full length of NF180 appears to be essential for the formation of thick NF bundles. In situ hybridization shows that like NF180, after spinal cord transection, expression of these newly discovered NFs first decreases, and then is recovered selectively in neurons whose axons have a high probability of regenerating.

MATERIALS AND METHODS

Construction of lamprey NFs

NF180 (Zhang et al., 2004) and the full-length cDNAs of three additional recently identified lamprey NF subunits were cloned and sequenced by either screening of a lamprey central nervous system (CNS) cDNA library or by PCR as described (Jin et al., 2011). They are named NF132, NF95, and L-NFL, based on their apparent size on SDS gels or by homology to mammalian NFL. The 9E10 antibody epitope (EQKLISEEDL) derives from a protein sequence in the human protooncogene p62 *c-myc* and is widely used as a protein fusion tag (Hilpert et al., 2001). To facilitate identification of individual NF subunits when performing single-transfection or cotransfection experiments, two sets of constructs were created for each cDNA; a wildtype (no tag added), and one with a *c-myc* tag fused to the amino-terminal by including the epitope sequence in the polymerase chain reaction (PCR) primers. The latter are named mycNF180, mycNF132, mycNF95, and mycL-NFL. To facilitate cloning, a sequence of restriction sites of HindIII and XbaI were also included in the 5' and 3' primers, respectively. The PCR product was ligated into pGEM-T Easy, a PCR cloning vector (Promega, Madison, WI) and subcloned into HindIII/ XbaI sites of pRc/CMV, a eukaryotic expression vector (Invitrogen, La Jolla, CA) used in our previous study (Zhang et al., 2004). For details of the cDNA sequences, please refer to National Institutes of Health (NIH) Gen-Bank accession numbers (L-NFL: DQ869188; NF95: DQ398934; NF132: DQ398933; NF180: U19361). The constructs were purified with a plasmid purification kit (Qiagen, Chatsworth, CA).

Construction of NF180 deletion mutants

In mammals, amino acid head and alpha-helical rod domains play important roles in NF assembly. In the present study only NF180, not NF132 or NF95, could form thick

filamentous bundles by coassembling with L-NFL. Therefore, to determine the molecular structural requirements for NF assembly we used PCR to generate four different deletion mutants of NF180, as shown in Figure 1A.

To construct NΔ82 (deletion of the first 82 of the 92 amino acids in the head domain, Fig. 1A), the full-length cDNA of NF180 was cloned into pBluescript II SK (Strata-gene, La Jolla, CACA) as a template. Then the following PCR primers were used. Forward: 5'-TTTaaagcttCACCATGAGCCAGTCGAGCGTGTT-3'; Reverse: T3 primer for the vector. A HindIII restriction site (lowercase letters in the above sequence) was added to the 5' primer. An XbaI site already exists in 3' of vector. The resulting 3,378 bp PCR product was ligated into pGEM-T Easy and subcloned into the HindIII/XbaI sites of pRc/CMV.

To construct CΔ314, CΔ586, and CΔ710, i.e., deleting 314, 586, and 710 residues from the carboxyl-tail domain, respectively (Fig. 1A), the following PCR primers were used. CΔ314: Forward: 5'-TTTaaagcttCACCATGAGC TACACAGAGTCG-3'; Reverse: 5'-ATTtctagaTATCAGATCTC GATGTCAAGCGCCAT-3'; CΔ586: Forward: 5'-TTTaaagcttCACCATGAGCTACACAGAGTCG-3'; Reverse: 5'-ATTtctagaTATCATTTCAGCTTCTTCCTCTGCTTC-3'; CΔ710: Forward: 5'-TTTaaagcttCACCATGAGCTACACAGAGTCG-3'; Reverse: 5'-ATTtctagaTATCATTTCTTAGCTTCAGGTGCAAC-3'. A stop codon (underlined) was added to the 3' primer in a reading frame for each construct. HindIII and XbaI restriction sites were added to the forward and reverse primers as indicated in lowercase letters. The resultant 2,388 bp, 1,572 bp, and 1,200 bp PCR products were ligated respectively into a pGEM-T Easy vector and then inserted into HindIII/ XbaI sites of pRc/CVM expression vector.

To facilitate detection, an additional set of deletion mutants with the myc tag attached to the amino-terminal was created by including the sequence of myc in the 5' primers in a correct open reading frame.

Cell culture and DNA transfections

Expression constructs encoding NF cDNAs were transfected into cell lines that lack endogenous cytoplasmic IFs in order to assess the structural requirements for assembly. Human adrenal carcinoma SW13cl.2Vim⁻ cells, which lack preexisting cytoplasmic IFs, were generously provided by Dr. Robert Evans (University of Colorado Health Science Center, Denver, CO). They were cultured in DMEM/F12 medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS) as described previously (Zhang et al., 2004). The cells were passed and grown on sterile glass coverslips coated with 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO) in 35-mm dishes at a density of 80% confluence on the day before transfection. The cells were transiently transfected with each myc tagged NF subunit construct, alone or in combination with other subunits, either myc-tagged or wildtype, using FuGENE6 transfection reagents (Roche Applied Science, Indianapolis, IN), following the protocol provided by the manufacturer. Assembly of fish vimentin is temperature-sensitive (Herrmann et al., 1993, 1996a, b; Herrmann and Aebi, 1999). To test the effect of temperature on lamprey NF assembly, after transfection the cells were cultured for the first 24 hours at 37°C, the appropriate temperature for a mammalian cell line, in order to maximize expression levels of the transfected proteins. This was followed by another 24 hours at 28°C, the optimal temperature for assembly of amphibian proteins (Bridger et al., 1998). Another group of samples was kept at 37°C until staining. The expression and assembly characteristics were examined by western blotting and immunofluorescent staining.

Antibody characterization (Table 1)

The specificity of the anti-vimentin antibody has been determined by demonstration of the appropriate 58-kDa band on immunoblots, using total cell extracts (manufacturer's datasheet). In this laboratory it stained only a small percentage of SW13cl.2Vim⁻ cells, due to contamination of the culture with vimentin-positive cells as reported previously (Osborn et al., 1984; Lee et al., 1993).

The *c-myc* antibody from Santa Cruz Biotechnology (Santa Cruz, CA; sc-789) was raised against amino acids 408 to 421 of human *c-myc* (Braiterman et al., 1998) and is used to detect *c-myc*-tagged fusion proteins (manufacturer's datasheet). It recognized only cells transfected with *c-myc*-tagged NFs but not nontransfected cells. On western blots of extracts from transfected cells, the *c-myc*-horseradish peroxidase (HRP) antibody recognized bands corresponding to each of the NF subunits, but recognized no proteins in extracts from nontransfected cells.

LCM3, a monoclonal antibody (mAb) raised in our laboratory and used in our previous article (Zhang et al., 2004), is a phosphate-independent antibody binding to the core of NF180, as determined previously by alkaline phosphatase digestion, chymotryptic digestion, and western blotting (Pijak et al., 1996). It recognized all truncated NF180 constructs in which most of the core region was retained, and also crossreacted with NF132 and NF95, but not with L-NFL (data not shown), as determined by western blotting of the recombinant peptides extracted from transfected cells.

Immunofluorescence

Cells transiently transfected with lamprey NFs were rinsed in phosphate-buffered saline (PBS, pH 7.4) and then fixed and extracted in cold methanol at -20°C for 10 minutes. After several washes with PBS the cells were blocked in PBS containing 3% bovine serum albumin (BSA) for 20 minutes and incubated with rabbit anti-*c-myc* primary antibody (1:200) overnight at 4°C. The cells were washed and incubated with secondary antibody of Alexa Fluor 594-conjugated (red) goat antirabbit IgG (A11012, 1:500, Molecular Probes, Eugene, OR) for 1 hour at room temperature. For double-labeling experiments, mycL-NFL was detected using a myc pAb and visualized with Alexa Fluor 594-conjugated goat antirabbit IgG, while vimentin or NF180 was detected using anti-vimentin (1:500) or LCM3 (1:100) mAb followed by a secondary antibody of Alexa Fluor 488 (green) goat antimouse IgG (A11029, 1:500 Molecular Probes). The coverslips were washed and mounted onto glass microscope slides with ProLong Gold antifade reagent (p36930, Invitrogen). Filaments were viewed on a fluorescence microscope with a 60× oil objective lens (Nikon, Eclipse 80i). The images were captured by digital camera (Photometrics, CoolSNAP EZ) and PhotoShop software (v. 7.0, Adobe Systems, San Jose, Ca) was used only to adjust the contrast and brightness of the images and to add labels.

Cell extraction and Immunoblotting

In order to verify the expression of truncated NF180 proteins the cells were harvested 48 hours after transient transfection with the myc-tagged construct of each deletion mutant 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 10 minutes prior to being loaded on the gels. As a control, nontransfected cell total homogenates were also obtained. 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 either monoclonal LCM3 (1:500) or HRP-conjugated myc antibody (R951, 1:5,000,

Invitrogen), which recognize 9E10 epitope, overnight at 4°C, washed extensively with 0.1M TBS containing 0.1% Tween-20. The blots with LCM3 were incubated with HRP-conjugated goat antimouse IgG secondary antibody at 1:3,000 (170–6516, Bio-Rad) for 1 hour. After washing with 0.1M TBS for 30 minutes, immunoreactive proteins were visualized with the enhanced chemiluminescence (ECL) kit (NEL103, PerkinElmer, Boston, MA) and exposed to x-ray film (Fig. 1 B, C) for varying amounts of time.

Wholemount in situ hybridization in SC transected lamprey brain

Hybridization of digoxigenin-labeled riboprobes to wholemounted lamprey brainstem was performed using techniques reported previously (Swain et al., 1994). Because mRNA expression level of NF180 in nontransected and transected lamprey brain had been investigated previously (Jacobs et al., 1997), and NF95 share all its DNA sequences with NF180, therefore the NF180 and NF95 were omitted. Furthermore, because the expression of all those NF subunits had been studied in normal lamprey brain and included in an article published (Jin et al., 2011) elsewhere, only expressions of L-NFL and NF132 in transected lamprey brain were included in this study.

Digoxigenin-labeled riboprobes were constructed based on alignment analysis, the most specific region of the genes were amplified by PCR (Jin et al., 2011), and ligated into pGEM-T Easy vector (Promega). For antisense probes synthesis, the vectors were linearized by restriction enzyme NcoI and in vitro transcription was performed with an RNA transcription kit as recommended by the manufacturer, with SP6 RNA polymerase in a 35:36 ratio of digoxigenin-11-UTP/unlabeled UTP.

Wildtype larval lampreys (*Petromyzon marinus*), 10–12 cm in length, were obtained from streams of Lake Michigan and maintained in freshwater tanks at 16°C until the day of transection. This work was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Lamprey spinal cords were transected under benzocaine anesthesia and the animals allowed to recovery for 2, 4, 6, and 10 weeks. A total of 15 animals were investigated: 3 at 2 weeks, 4 at 4 weeks, 5 at 6 weeks and 3 at 10 weeks. The animals were reanesthetized and brainstems removed, pinned flat on Sylgard strips, and fixed in 4% paraformaldehyde for 3–4 hours. Wholemounted brainstem preparations were washed in PTW (0.1% Tween 20 in PBS) and prehybridized at 55°C in hybridization solution (50% deionized formamide, 5 × SSC, 100 pg/ml *Torula* yeast RNA, 100 pg/ml wheat germ tRNA, 50 pg/ml heparin, 0.1% Tween-20) followed by hybridization overnight at 55°C in the same solution plus 400 ng/ml digoxigenin-labeled probes of L-NFL and NF132. Specimens were washed in hybridization solution at 55°C followed by room temperature washes in PTW and PBT (0.1% bovine serum albumin, 0.2% Triton ×-100 in PBS). Alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1,000; Roche, Nutley, NJ) was applied to tissue overnight at 4°C. The tissue was washed sequentially in PBT and SMT (100 mM NaCl, 50 mM MgCl₂, 100 mM Tris, pH 9.5, 0.1% Tween-20) and the chromogenic reaction was carried out in ice-cold SMT containing 175 pg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 350 pg/ml 4-nitro blue tetrazolium chloride on ice in the dark. Finally, specimens were washed in PBS, dehydrated in serial ethanols, cleared with xylene, and mounted with Permount.

RESULTS

Temperature-dependent assembly of lamprey NFs

Whereas most mammalian enzymatic reactions increase their activities with increasing temperature up to 40°C or more, enzymes of poikilotherms may function better at lower temperatures. For example, assembly of *Xenopus* vimentin occurs optimally at 28°C

(Herrmann et al., 1993). To examine the effect of temperature on assembly of lamprey NFs, L-NFL and NF180 were first cotransfected into SW13cl.2Vim⁺ cells, a mammalian-derived cell line. After incubation for 24 hours at 37°C the cells were divided into two groups. One remained at 37°C, while the other was shifted to 28°C for another 24 hours. At 37°C, nonfilamentous aggregates were observed in nearly all cells. In most cells, short, disconnected filamentous fragments were observed (arrow in Fig. 2A). When protein expression levels were high, the punctuate, nonfilamentous aggregates became confluent (cell in lower right of Fig. 2A). However, extensive filamentous arrays appeared when the transfected cultures were transferred to 28°C (Fig. 2B). Thus, assembly of lamprey NFs is temperature-dependent, occurring more efficiently at 28°C than at 37°C, even when occurring in mammalian cells.

Myc tag in the amino-terminal domain did not affect filament assembly

Peptide-tagging permits assessment of interactions between the labeled protein and other subunits, eliminates antibody crossreactivity with similar IFs, and distinguishes transfected from endogenous NFs in *in vivo* studies. Many NF transfection experiments have used similar tagging of epitopes and found it did not affect assembly when the conjugation is in the carboxyl-terminal. To simplify cloning we cloned the myc tag into the amino-terminal. Since part of the head domain plays a critical role in NF assembly, an effect of adding the N-terminal tag on filament assembly was ruled out by transfecting mycL-NFL with wildtype NF180, wildtype L-NFL with mycNF180, and wildtype L-NFL with wildtype NF180 into SW13cl.2Vim⁺ cells as described above and the effect on filament assembly observed. The first two combinations were stained with the anti-myc antibody to image the tagged proteins and the third combination was stained with LCM3 to image NF180. All combinations formed typical filamentous networks and no differences in staining pattern were seen, indicating that assembly of NFs was not interrupted by adding the myc tag at the N-terminus.

Individual lamprey NF subunits are unable to self-assemble

To determine the ability of each lamprey NF subunit to self-assemble, myc-tagged full-length cDNA of L-NFL, NF95, NF132, or NF180 was introduced singly into SW13cl.2Vim⁺ cells. Transfected cells were stained with anti-myc antibody and visualized with Alexa Fluor 594-conjugated secondary antibody. None of the lamprey NF proteins could self-assemble into filament networks either at 37°C or 28°C (Fig. 3). Cells transfected with L-NFL often exhibited a punctate staining pattern (Fig. 3A), while cells transfected with all other subunits showed only a diffuse staining pattern.

Coassembly properties of L-NFL, NF180, NF132, and NF95

In the mammalian system it has been reported that NFL coassembles with either NFM or NFH to form filamentous structures in cells lacking endogenous IFs, indicating that NFL is key in NF assembly. To determine the assembly properties of lamprey NF subunits, coexpressions of mycL-NFL with each wildtype subunit were accomplished and stained by anti-myc antibody. As described above, coassembly was performed at two different temperatures, 37°C and 28°C. At 37°C (data not shown), expression of L-NFL alone yielded a punctuate staining pattern, and expression of L-NFL with all other subunits resulted in nonfilamentous aggregates in most cells, although occasionally some short, filamentous fragments were seen. When transfected cells were shifted from 37°C to 28°C, L-NFL was still unable to self-assemble (Fig. 4A), but could form filaments if cotransfected with any one of the other subunits, NF95, NF132, or NF180 (Fig. 4B – D). Thus, at the correct temperature L-NFL can coassemble with all of the lamprey NFM-like subunits. However, whereas NF95 or NF132 formed only densely packed thin filamentous networks with L-NFL (Fig. 4B,C), NF180 often polymerized with L-NFL to form thicker cable-like

filamentous bundles (Fig. 4D). Double labeling with the monoclonal antibody LCM3 for NF180 and the polyclonal anti-myc antibody for mycL-NFL revealed colocalization of those two molecules, indicating that they were bound to each other (Fig. 5). As reported previously, a small percentage of SW13cl 2Vim[−] cells spontaneously revert to a vimentin-positive phenotype (Lee et al., 1993). In mammalian systems, vimentin can coassemble with NF (Sun et al., 1997). In order to confirm that the observed filaments were truly due to coassembly of L-NFL with other lamprey NF subunit and not with preexisting vimentin, the cells transfected with mycL-NFL and NF180 were double-stained with a monoclonal antibody for vimentin and a polyclonal anti-myc antibody for L-NFL. As indicated in Figure 6, L-NFL and NF180 coassembled in vimentin-negative cells (arrowhead).

To determine whether NF95 or NF132 also could be incorporated into thicker bundles in the presence of L-NFL plus NF180, triple transfections were performed. In order to label NF95 or NF132 specifically, mycNF95 or mycNF132 was transfected with untagged wildtype L-NFL and NF180 and immunostained with the anti-myc antibody. As shown in Figure 7, both NF95 and NF132 were incorporated with L-NFL plus NF180 into filament bundles. To determine whether any of the NF subunits other than L-NFL could serve as the obligatory subunit in filament assembly, SW13cl 2Vim[−] cells were transfected with various combinations of NF subunits as indicated in Figure 8, i.e., NF95 with either NF132 or NF180, NF132 with NF180, and triple transfections of all those three subunits. None of those combinations could make filaments, confirming that only L-NFL has the ability to coassemble with other subunits into filamentous networks.

Sequences within the amino-terminal head and core of NF180 are essential for assembly but the carboxyl-terminal is essential for normal network appearance

NF180 is the largest of the three lamprey NFM-like subunits, and is the only subunit that formed filamentous bundles with L-NFL. To study the influence of the various regions of NF180 on filament assembly and bundling, we generated one amino- and three carboxyl-terminal deletion mutants as depicted in Figures 1A and 9A. When up to 586 amino acid residues (almost the entire sidearm) were removed from the tail domain, NF180 retained the ability to coassemble with L-NFL into filamentous networks (Fig. 9E,F). This indicated that the tail domain was dispensable for coassembly with L-NFL into the filaments. However, carboxyl-terminal deletion into the alpha-helical rod domain resulted in punctuate structures similar to the staining pattern seen when L-NFL was transfected alone (Fig. 9D). Deletion of 82 amino acid residues from amino-terminal head domain resulted in an inability of NF180 to coassemble with L-NFL into filaments (Fig. 9C). As indicated in Figure 9G, the entire carboxyl-terminal tail domain of NF180 was required for effective bundling of NFs.

NF mRNA expression in axotomized reticulospinal neurons

Changes in expression of the newly discovered NFs were analyzed qualitatively by in situ hybridization at 2, 4, 6, and 10 weeks after spinal cord transection. The expression patterns and responses to axotomy were similar to those described previously for NF180 (Jacobs et al., 1997). At 2 weeks posttransection, only a few reticulospinal neurons were swollen and had lost NF mRNA expression (arrows). NFs mRNA levels in most neurons appeared close to normal (Fig. 10A,D). By 6 weeks after transection, almost all axotomized reticulospinal neurons were swollen and had dramatically reduced levels of NF mRNAs (Fig. 10B,E). However, the depression of NF mRNA levels in “good regenerators” (arrows in Fig. 10B,E) appeared less than in “bad regenerators” (Jacobs et al., 1997). Ten weeks after transection, NF mRNA levels remained depressed in bad regenerator neurons as indicated by arrows in Figure 10C.F (B1, B3, B4, and Mth). By contrast, the neurons that have a high probability of axonal regeneration recovered NFs expression (arrowheads, B2, B5, B6 and mth'). Similar changes were found for both L-NFL and NF132.

DISCUSSION

Lamprey NFs have multiple subunits

It had been thought that lamprey NFs contain only one subunit, i.e., NF180, which was most similar to human NFM but combined features of all three mammalian NF proteins, NFL, NFM, and NFH (Pleasure et al., 1989; Jacobs et al., 1995). It was therefore assumed that NF180 self-assembles into NFs. (This would have provided a convenient model for testing the role of NF in axonal regeneration.) However, more recent results indicated that NF180 lacks the ability to self-assemble but could be incorporated into typical filamentous networks in the presence of rat NFL plus NFM (Zhang et al., 2004). Furthermore, we found evidence for an additional neuronal IF that migrated at 50 kDa on SDS gels (Jin et al., 2005). In an (as yet unsuccessful) attempt to clone this subunit, we found three additional NF subunits, two NFM-like named NF95, NF132, and one homologous to NFL (L-NFL).

Lamprey NFL does not self-assemble

Although under some conditions mammalian NFL can assemble into filaments in vitro (Geisler and Weber, 1981; Liem and Hutchison, 1982), in vivo NFL requires NFM or NFH to form typical filamentous networks (Lee et al., 1993). We do not know if L-NFL also can assemble into filaments in vitro. However, in cell culture L-NFL alone aggregated into punctuate structures, and sometimes into confluent sheet-like structures, similar to the appearances of NF95, NF132, or NF180 when they are expressed individually. Thus, as with mammalian NFL, L-NFL does not form filaments by itself when expressed inside cells.

L-NFL is a necessary constituent of NFs in lamprey

None of the three NFM-like subunits of lamprey were able to form filaments by themselves or in any combination. However, all three NFM-like subunits could be incorporated into filaments in the presence of L-NFL. Thus, as in mammals, in lampreys L-NFL is a necessary constituent of NFs.

Of interest, only NF180 could form filamentous bundles with L-NFL. NF95 and NF132 could form only thin, wispy filaments. The molecular basis for the inability of NF95 and NF132 to form robust bundles is not known. However, the three NFM-like subunits are highly homologous to each other, differing mainly in the length of the tail domain, with NF180 being the longest. This together with evidence that truncated NF180 that is missing only the carboxyl-terminal tail behaves similarly (see below), suggests that bundling is a function of the long carboxyl-terminal tail domain of NFs.

Temperature-dependence of NF assembly

NF assembly has been much less well studied in fish and other nonmammalian species than in mammals. Previous studies have suggested that IF assembly is temperature-dependent and that in poikilotherms cells must be grown at lower temperatures than the conventional 37°C used to study most processes in mammalian systems. For example, polymerization of *Xenopus* vimentin in a bovine mammary gland-derived cell line occurred most effectively at 28°C, decreasing by ≈30% at 22°C, by ≈50% at 31°C, by ≈75% at 34°C, and by ≈90% at 37°C (Herrmann et al., 1993). Trout vimentin requires even colder temperatures (Herrmann et al., 1996b). This might explain findings that suggested functional differences between homologous molecules of mammals and non-mammalian species. For example, plasticin is a type III IF found in zebrafish. In contrast to its mammalian ortholog peripherin, plasticin was unable to form a homopolymeric IF network in SW13cl.2Vim⁻ cells (i.e., mammalian cell line lacking an endogenous IF cytoskeleton), suggesting that there are important structural differences in assembly properties between fish IF proteins and their mammalian

counterparts (Asch and Schechter, 2000). These experiments were carried out by growing the cells at either 37°C or 32°C. It is not known whether growing them at even colder temperatures, such as 28°C, as in the present study, would have yielded other results.

In the current study, none of the NFM-like subunits could assemble with L-NFL at 37°C. In differentiated NB21/d1 neuroblastoma cells, full-length NF180 could coassemble with endogenous NFs into filamentous networks (Hall et al., 2000; Lee et al., 2008). Yet in those studies the NB21/d1 cells were cultured at 37°C. Interestingly, our previous study had also shown that at 37°C NF180 could be incorporated into filaments, even when expressed in cells lacking endogenous IFs, as long as it was coexpressed with mammalian NFL plus NFM. Thus, it may be that although the NF subunits of poikilotherms cannot serve as the essential large subunit to bind to NFL in order to form filaments, the poikilotherm long NFs can be incorporated into filaments at 37°C as long as a long mammalian NF subunit is present to play that essential role.

The present results confirm the temperature sensitivity of assembly of poikilothermic NFs. Because the cell line used in the present study is derived from mammals, its physiological temperature is 37°C. Nevertheless, the lamprey NF subunits assembled into filamentous arrays only after cells were transferred to 28°C. This indicates that the temperature sensitivity of IF assembly is a consequence of sequence variations in the IF proteins themselves, and not of the enzymatic machinery of the host cells.

Molecular basis of NF assembly

NFs consist of 10-nm diameter filaments, known as “core filaments,” and cross-bridges that stick out between each pair of filaments. The cross-bridges are thought to maintain the spacing between filaments and regulate axon caliber. In mammals, NFL forms the core of NFs by binding with the alpha-helical rod domains of NFM and/or NFH, while the carboxyl terminal domains of NFM and/or NFH are involved in cross-bridge formation (Hisanaga and Hirokawa, 1988). Deletion experiments on mammalian NFs have elucidated the roles of the amino and carboxyl terminals in NF protein assembly (Gill et al., 1990; Wong and Cleveland, 1990; Ching and Liem, 1993; Sun et al., 1997). The results suggested that the head and alpha-helical core play important roles in filament formation, but the carboxyl terminal does not. Mouse NFM missing up to 90% of the nonhelical carboxy-terminal tail or 70% of the amino-terminal head domain could be incorporated into filaments with either NFL or vimentin (Wong and Cleveland, 1990). Similar observations were reported for rat NFH (Sun et al., 1997). The present findings in lamprey are generally consistent with these mammalian studies.

Importance of the amino head domain—Deletion of 82 residues (84%) of the NF180 amino-terminal head region but leaving the rod domain intact disrupted filament assembly. In mouse NFM, removing 75 residues (60% of head domain) from amino-head domain did not disrupt assembly, but deletion into rod domain resulted in assembly incompetence (Wong and Cleveland, 1990). The authors did not pinpoint more precisely where the disruption occurred, but the mammalian studies are consistent with the present findings that a region critical for neurofilament assembly may exist at a point between 60 and 84% from the amino terminus to the core.

Importance of the carboxy-terminus sidearm—To determine the role of the carboxyl-terminal tail domain of mammalian NFM on NF assembly, five deletion mutants of NFM lacking different lengths of the tail domain were constructed and coexpressed with NFL in nonneuronal insect Sf9 cells, which lack endogenous IFs (Nakagawa et al., 1995). Quick-freeze, deep-etch electron microscopy revealed that all deletion mutants could

coassemble with NFL into 10-nm filaments. However, filaments coassembled by NFL and the mutant lacking most of the tail domain lacked cross-bridges, and cross-bridges formed by coassembly of NFL with other mutants were shorter and less frequent compared to those formed by wildtype NFM, and thus the tendency of filaments to develop parallel coordination (i.e., to form bundles) became stronger as the length of carboxyl-terminal tail domain increased.

The above view is generally supported by the results of the present study. The three NFM-like subunits in the lamprey vary in length, and only the longest, i.e., NF180, could form thick filament bundles. As in mammalian NFM and NFH, the sidearm of NF180 consists of a proximal proline-rich region, an intermediate glutamate-rich region, and a distal region containing multiple potential phosphorylation sites, although it lacks the typical KSP repeats of mammalian NFM and NFH (Jacobs et al., 1995). Expression of truncated forms of rat NFL and NFM in fibroblasts revealed that only deletions of the carboxyl-terminal that extended into the alpha-helical rod domain resulted in an inability of the mutant proteins to coassemble with endogenous vimentin (Chin et al., 1991). Experiments in which truncated lamprey NF180 was transfected into neuroblastoma cells gave a somewhat different result. In differentiated NB21 /d 1 neuroblastoma cells, coassembly of NF180 with endogenous NFs was disrupted by deletion of the glutamate-rich region of NF180 (Lee et al., 2008). However, studying the assembly properties of truncated nonmammalian NFs together with mammalian NFs may yield misleading results. Moreover, network incorporation of IF differs between preexisting and newly assembling filaments (Lu et al., 1993). In our previous study using cell lines that lack endogenous IFs, NF180 did not coassemble with mammalian NFL to form normal filaments, but formed only thick rod-like structures, both at 37°C and 28°C (Zhang et al., 2004). More normal-looking filaments were formed when NF180 coassembled with mammalian NFL plus NFM, which more closely reproduces the results in differentiated neuroblastoma cells. In the present study, deletion of the carboxyl tail of NF180 through the glutamate-rich region did not affect the formation of filaments. These results suggest that the glutamic acid-rich portion of the carboxyl-terminal tail does not play a critical role in filament assembly. However, none of the truncated NF180 mutants formed thick filament bundles with L-NFL, although NF180 truncated within the carboxyl terminal tail formed wispy, disordered filaments. Similar structures were seen when L-NFL was coassembled with the two shorter NFM-like subunits, NF95 and NF132.

Possible role of NFs in axonal regeneration

The evolution and function of NFs were described by Lasek and others (Phillips et al., 1983; Lasek et al., 1985). Although NFs have been viewed as relatively static structures that play a role in determining axon caliber, some evidence suggests a more active role in axon regeneration. In mice lacking NFs the regeneration of myelinated axons following crush injury of peripheral nerves was found to be abnormal (Zhu et al., 1997). The number of newly regenerated myelinated axons in the sciatic nerve and facial nerves of NFL knockout mice corresponded to only 25% and 5% of the number of myelinated axons found in normal mice, respectively. A correlation was found between the regenerative capacities of reticulospinal neurons and their ability to resume expression of lamprey NF mRNAs after an initial downregulation after spinal cord transection. Labeling for all NF messages was reduced in all reticulospinal neurons early after spinal cord transection. However, after several weeks, expression of the newly discovered NF genes recovered only in neurons whose axons regenerate frequently. Those results were similar to previous findings for the NF180 gene (Jacobs et al., 1997). This finding, together with the prominence of NFs in the regenerating axon tips, the paucity of actin microfilaments, the absence of lamellipodia and filopodia (Lurie et al., 1994; McHale et al., 1995; Pijak et al., 1996; Hall et al., 1997), and the finding that the pattern of NF reexpression was maintained even if regeneration was

prevented mechanically (Jacobs et al., 1997), suggested the hypothesis that NFs may be involved in the mechanism of axon regeneration (Lurie et al., 1994; Pijak et al., 1996; Hall et al., 1997; Jacobs et al., 1997). Moreover, because the good regenerators and their axons are located side by side with the bad regenerators, it is unlikely that environmental differences underlie the heterogeneity of regenerative capacities of the identified reticulospinal neurons. Thus, it was suggested that the reexpression of NF180 might be part of an intrinsic regeneration program, and was not simply a consequence of axon regeneration (Jacobs et al., 1997). If so, then it would be expected that a similar effect of axotomy might be seen in the expression of the newly cloned NFs, particularly L-NFL, which was absolutely required to make NFs. However, other interpretations are possible. Recently, it was shown that some of the “badregenerating” neurons undergo very delayed apoptosis. This suggests that part of what makes a neuron a “bad regenerator” is that by the time it is assessed for axon regeneration it is already in the process of dying (Shifman et al., 2008).

CONCLUSIONS

The results of this study suggest that the NFs of lampreys behave more like those in mammals than was believed previously. As in mammalian NFs, L-NFL is necessary for the formation of filaments, but this requires the presence of one of the longer NF subunits in vivo. As in mammals, a sequence somewhere between 60 and 84 amino acids from the amino-terminus of the head domain of NF180 is critical for NF assembly. And as in mammals, the carboxy-terminus tail domain of NF180 is not required for NF assembly, but is involved in organizing the filaments into thicker bundles. Some of the apparent differences between lamprey and mammalian NFs can be ascribed to three factors. First is the previous failure to find a lamprey NFL (Lasek et al., 1985; Pleasure et al., 1989; Jacobs et al., 1995). Together with immunochemical and sequence data that suggested that NF180 combines essential features of all three mammalian subunits (Pleasure et al., 1989; Jacobs et al., 1995), this gave rise to the expectation that NF180 was the only lamprey NF subunit and that therefore it must be capable of self-assembly. Second is the temperature-dependence of NF assembly in poikilotherms, which results in aberrant behavior of NFs at temperatures conventionally used in culturing mammalian cells. Third is the ability of lamprey NF180 to attach to (or be incorporated into) mammalian NFs that have been preassembled or that can assemble because the usual conditions for assembly are present. Lamprey NFs differ from those in mammals in several ways. Among these is the absence of a stereotyped KSPA/V phosphorylation repeat in the carboxy-terminus tail of the long NF subunits, although there are numerous potential phosphorylation sites (Jacobs et al., 1995), and both phosphorylated and unphosphorylated forms of NF180 exist in vivo (Pleasure et al., 1989). Second is the presence of at least three NFM-like proteins. Since only NF180 is capable of promoting the organization of NFs into thicker bundles, this raises the question of what function the shorter NFM-like subunits (NF95 and NF132) serve in vivo. A third apparent difference is the absence to date of a lamprey NFH homolog. However, there appear to be additional NF sequences in the genomic database that have not been assembled, and it may be that an NFH will yet be found. Finally, the mRNA levels of all NF subunits were severely reduced after spinal cord transection, but recovered only in neurons whose axons normally regenerate well. Thus, the results of this study are consistent with the hypothesis that NFs play a role in the mechanism of axonal regeneration.

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Figure 1.

Truncated NF180 Constructs. **A:** Schematic illustration of full-length (modified from Jacobs et al., 1995) and truncated NF180 constructs utilized in this study. The encoded amino acid numbers are shown below each truncated NF180 structure and their nomenclatures as indicated herein. **B,C:** Immunoblot detection of truncated NF180 constructs following transient transfection into SW13c1.2Vim⁻ cells. Protein extracts from cells transfected with the full-length and each truncated NF180, respectively, were probed using the anti-NF180 antibody LCM3 (binds to the N-terminal of NF180). Molecular markers in kilodaltons are indicated at the left. **C** is the continuation of the gel in **B** showing peptides smaller than 80 kD. All constructs (arrows) appeared at their appropriate molecular weights.

Figure 2.

L-NFL can form filaments with NF180 at 28°C but not at 37°C. Immunofluorescence of SW13cl.2Vim⁻ cells transiently trans-fected with mycL-NFL plus NF180 and cultured at either 37°C (**A**) or 28°C (**B**). The cells were stained with a polyclonal antibody to myc and followed by a secondary antibody of Alexa Fluor 594-conjugated donkey antirabbit IgG. Scale bar = 20 μ m.

Figure 3.

Individual lamprey NF subunits cannot self-assemble in cells lacking endogenous IFs at optimal temperature (28°C). The myc-tagged full-length cDNA of each indicated subunit was transiently transfected into SW13c1.2Vim⁻ and stained with a polyclonal antibody to myc followed by Alexa Fluor 594-conjugated donkey antirabbit IgG. Scale bar = 20 μ m.

Figure 4.

Coassembly of L-NFL with individual NFM-like peptides. Immunofluorescence of SW13cl.2Vim⁻ cells transiently transfected with mycL-NFL alone (**A**) or with NF95 (**B**), NF132 (**C**), or NF180 (**D**) and cultured at 28°C. The cells were stained with a polyclonal antibody to myc followed by Alexa Fluor 594-conjugated donkey antirabbit IgG. L-NFL was unable self-assemble but coassembled with each NFM-like subunit. However, long, thick bundles were formed only with NF180. Scale bar = 20 μ m.

Figure 5.

Coassembly of L-NFL and NF180 into thick filamentous networks. SW13cl.2Vim⁻ cells transiently transfected with mycL-NFL and wildtype NF180 were doubly stained with polyclonal anti-myc antibody for L-NFL (A) and monoclonal antibody LCM3 for NF180 (B), followed by secondary antibodies Alexa Fluor 594-conjugated donkey antirabbit IgG (red) and Alexa Fluor 488-conjugated goat antimouse IgG (green). (C) L-NFL and NF180 staining superimposed, indicating colocalization. Scale bar = 20 μ m.

Figure 6.

Filaments containing L-NFL plus NF180 are not due to coassembly with vimentin. A small percentage of SW13cl.2Vim⁻ cells revert to vimentin positivity. Therefore, SW13cl.2Vim⁻ cells transiently transfected with mycL-NFL and wildtype NF180 were doubly stained with polyclonal anti-myc and monoclonal anti-vimentin antibodies, followed by secondary antibodies Alexa Fluor 594-conjugated donkey antirabbit IgG (red) (A) and Alexa Fluor 488 goat antimouse IgG (green) (B), respectively. (C) is (A,B) merged. Insert in A is an enlargement of the labeled cell indicated by an arrowhead, showing a typical filamentous network. Arrows in (B) indicate a small group of cells that had reverted to vimentin positivity. There is no vimentin in the NF-positive cell. Scale bar = 20 μ m.

Figure 7.

NF95 or NF132 can be incorporated into thicker filamentous bundles when tri-transfected with L-NFL plus NF180. SW13c1.2Vim⁻ cells transiently transfected with mycNF95 (**A**) or mycNF132 (**B**) and wildtype L-NFL and NF180 were stained with a polyclonal antibody to myc followed by the secondary antibody Alexa Fluor 594-conjugated donkey antirabbit IgG. Scale bar = 20 μ m.

Figure 8.

NFM-like subunits do not form filaments in the absence of L-NFL. Immunofluorescence of SW13cl.2Vim⁻ cells transiently transfected with various combinations of NF 95, NF132, and NF180 as indicated. The cells were cultured at optimal temperature 28°C and stained with the LCM3 antibody followed by secondary antibody Alexa Fluor 488-conjugated goat antimouse IgG. None of the NF combinations made filamentous structures. Scale bar = 10 μm.

Figure 9.

The full length of NF180 is required for normal filament bundle formation. Schematic of the NF180 protein (A) and a DNA agarose gel (B) showing fragments of truncated NF180 removed with HindIII/XbaI restriction enzymes from the eukaryotic expression vector pRc/CMV (5.5 kb indicated by arrow). SW13cl.2Vim⁻ cells from cotransfections of myc-L-NFL with NΔ88 (C), CΔ710 (D), CΔ586 (E), CΔ314 (F), and full-length NF180 (G) were stained with a polyclonal antibody to myc followed by the secondary antibody Alexa Fluor 594-conjugated donkey antirabbit IgG. Note that sequences within the amino-terminal head and rod domains, but not the carboxyl-terminal tail of NF180 are essential for coassembly with L-NFL to form fine filaments, but the full length of NF180 is necessary to form thicker NF bundles. Scale bar = 20 μm.

Figure 10.

Recovery of NFs mRNA expression selectively in “good regenerating” reticulospinal neurons. **A–F:** The right middle rhombencephalon in the region of several large identified reticulospinal neurons (as labeled). In situ hybridization was performed on wholemounted brainstems of transected animals 2 weeks (A,D), 5 weeks (B,E), and 10 weeks (C,F) after spinal cord transection at the 5th gill. Note that NF message levels in axotomized reticulospinal neurons were not reduced from control levels (not shown) during the first 2 weeks (A,D) in most neurons but was dramatically reduced by 5 weeks (B,E) after axotomy. In the poorly regenerating neurons (B1,B3,B4 and Mauthner, white arrows), this reduced expression was sustained by 10 weeks after spinal cord transection. By contrast, in the neurons that frequently regenerate (e.g., B2,B5,B6, and mth’, black arrowheads), NF message levels showed at least partial recovery by 10 weeks after axotomy. Scale bar = 50 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1

List of Primary Antibodies

Antigen	Immunogen	Manufacturer	Dilution
Vimentin	pig eye lens vimentin (antigen molecular weight ~58 kDa)	Sigma-Aldrich, monoclonal antibody, clone V9, Isotype mouse IgG, Cat. no. V6630	1:500
<i>c-myc</i> (A-14)	a peptide corresponding to aa 408–421 of human <i>c-myc</i>	Santa Cruz, polyclonal antibody developed in rabbit, Cat. no. sc-789	1:200
Neurofilaments (lamprey)	cytoskeletal proteins of lamprey CNS (recognizes NF180, NF132 and NF95)	Selzer lab, monoclonal antibody developed in mouse clone 3	1:100
<i>c-myc</i> -HRP	recombinant proteins containing the <i>c-myc</i> epitope "Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu"	Invitrogen, Cat. no. R951–25	1:5,000