Neurofilaments Are Obligate Heteropolymers In Vivo

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Abstract. Neurofilaments (NFs), composed of three distinct subunits NF-L, NF-M, and NF-H, are neuron-specific intermediate filaments present in most mature neurons. Using DNA transfection and mice expressing NF transgenes, we find that despite the ability of NF-L alone to assemble into short filaments in vitro NF-L cannot form filament arrays in vivo after expression either in cultured cells or in transgenic oligodendrocytes that otherwise do not contain a cytoplasmic intermediate filament (IF) array. Instead, NF-L aggregates into punctate or sheet like structures. Similar nonfilamentous structures are also formed when NF-M or NF-H is expressed alone. The competence of NF-L to assemble into filaments is fully restored by coexpression of NF-M or NF-H to a level ~10% of that of NF-L. Deletion of the head or tail domain of NF-M or substitution of the NF-H tail onto an NF-L subunit reveals that restoration of in vivo NF-L assembly competence requires an interaction provided by the NF-M or NF-H head domains. We conclude that, contrary to the expectation drawn from earlier in vitro assembly studies, NF-L is not sufficient to assemble an extended filament network in an in vivo context and that neurofilaments are obligate heteropolymers requiring NF-L and NF-M or NF-H.

In most eukaryotic cells, the cytoskeleton is composed of three distinct structural components with characteristic diameters: actin-microfilaments (7 nm), intermediate filaments (IFs) (8-10 nm), and microtubules (24 nm). The subunits from which cytoplasmic and nuclear IFs are assembled have been categorized into five distinct groups based on primary amino acid sequence differences and the intron positions of the genes encoding the subunits (Steinert and Roop, 1988). Types I and II include acidic and basic keratins expressed primarily by epithelial cells. The type III consists of vimentin expressed in cells of mesenchymal origin, desmin expressed in muscle cells, GFAP expressed in astrocytes, and peripherin expressed by a subset of neurons in the peripheral nervous system. Type IV consists of neurofilaments (NFs) expressed by most mature neurons, although nestin, expressed in neural precursor cells and α-internexin, expressed in subset of neurons particularly during early neurite outgrowth, probably belong to type IV as well (Fliegner et al., 1990; Cleveland et al., 1991). The nuclear lamins that form the filaments underlying the nuclear membrane constitute type V. All of these IF proteins are characterized by an α-helical domain of ~310 (or 352 for the lamins) amino acids that is flanked by globular amino-terminal head and carboxyl-terminal tail domains whose lengths vary and whose amino acid sequences are not conserved.

1. Abbreviations used in this paper: CMV, Cytomegalovirus; GFAP, glial fibrillary acidic protein; IF, intermediate filament; MSB, microtubule stabilization buffer; MSV, murine sarcoma virus; NF, neurofilament.

In most mature neurons, particularly in the neurons that give rise to large myelinated axons, the IF system is almost exclusively composed of neurofilaments. In these axons, neurofilaments are by far the most abundant cytoskeletal element, often exceeding the number of microtubules by an order of magnitude. Several lines of evidence have collectively demonstrated that proper assembly and organization of neurofilaments in axons are important for specifying the axonal diameter of the large caliber myelinated axons (see Cleveland et al., 1991; Yamasaki et al., 1991). More recently, transgenic mice models have shown that disruption of normal neurofilament subunit content by over-expression of either NF-L or NF-H results in abnormal assembly and aggregation of neurofilaments in neuronal cell bodies, particularly in the motor neurons (Côté et al., 1993; Xu et al., 1993). In these transgenic mice, the disruption of normal organization of neurofilaments was accompanied by motor neuron defects that ultimately resulted in denervation-induced atrophy of skeletal muscles. The role of neurofilaments in these pathogenic events highlights the importance of identification and characterization of domains in NF subunits that participate in normal cytoplasmic assembly and organization.

Unlike most other non-epithelial IFs, which are homopolymers (e.g., vimentin, desmin, and GFAP), neurofilaments in mature neurons are assembled from three separate subunits called NF-L, NF-M, and NF-H, with true molecular masses of ~60 kD (NF-L), ~100 kD (NF-M), and ~120 kD (NF-H) (reviewed by Nixon and Shea, 1992). The presence of a conserved 310 amino acid α-helical rod domain on
corporated into filaments in the presence of NF-L, indicating Type I/II filaments (keratin) and yields short, somewhat ir-
to the reassembly of either Type III filaments (vimentin) or
Thus, potential functional roles for both NF-M and NF-H
rofilaments are probably assembled from an NF-L core and
The encoded amino acid numbers are shown below the
drawn to scale and the actual size of introns are indicated. (D)
promoter described below. A 600 base (HindIII-BglII fragment) containing
used to create HindIII restriction sites in both NF-L and NF-H genes
For NF-H, a 2.3-kb EcoRI fragment (encoding amino acids 267-1053 of
which ends in a sequence (RKLLEGE) common to both NF-L and NF-H.
and at exactly the same position in the carboxy-terminal portion of the rod,
and the ATG translation initiation codon and blunted with Klenow and
dNTP. The DNA was then digested with HindIII and the 5-kb NF-L gene
fragment missing the 5'-promoter region was isolated. To produce pCMV, the
600-bp human CMV early promoter was cloned between KpnI and XhoI sites in pGEM-3z. To produce pCMV-NF-L, pCMV was digested with
XhoI, blunted with Klenow and digested with HindIII. The 5-kb 5'-blunt
3'-HindIII NF-L fragment from above was then ligated into blunt/HindIII sites in the 3' CMV promoter.
To construct pMSV-NF-H (Fig. 1 C), a 12-kb fragment containing the mouse NF-H gene was obtained by double digesting Cos3Al (kindly provided by Dr. J.-P. Julien, Montreal, Canada; Julien et al., 1988) with KpnI and SalI. To obtain pNFH, the 12-kb NF-H gene was cloned between KpnI and SalI sites of pUC19. To obtain pMSV-NF-H, the NF-H promoter in pNFH was removed using KpnI and NotI and replaced with the MSV promoter described below. A 600 base (HindIII-BglII fragment) containing the MSV promoter was subcloned between the HindIII-BamHI sites of pMSV-NF-L to generate pMSV-NF-H. Oligonucleotide-directed mutagenesis was used to create HindIII restriction sites in both NF-L and NF-H genes and at exactly the same position in the carboxy-terminal portion of the rod, which ends in a sequence (RKLEGE) common to both NF-L and NF-H. The nucleotide changes did not alter the amino acid sequence (Fig. 1 D). For NF-H, a 2.3-kb EcoRl fragment (encoding amino acids 267-1053 of NF-H) from a partial NF-H cDNA clone, pMuHII (kindly provided by Dr. P. Shneidman, University of Pennsylvania; Shneidman et al., 1988), was subcloned into the EcoRl site of M13mpl8. Single-stranded DNA was obtained from a clone with desired orientation and a HindIII site was engineered into the gene by oligonucleotide-directed mutagenesis using the Amersham kit, version 2.1 (Amersham Corp., Arlington Heights, IL). The mutants were confirmed by DNA sequencing. Double-stranded DNA was recovered and digested with HindIII and EcoRV. The mutated 1.3-kb fragment was cloned between the EcoRl-HindIII sites of pBSII-KS* to produce pMuHII(H3)-BS. To introduce a HindIII site in the NF-L gene, 1 kb KpnI fragment containing the sequences encoding the terminal rod domain (including the KLLEGE) was subcloned into the KpnI site of pBSII-KS*. Single stranded DNA templates were rescued (using helper phage R408)

Figure 1. Gene constructs for expression NF-L, NF-M, NF-H, and LLH-hybrid subunits. (A) Schematic drawing of CMV-NFL or pMSV-NFL genes. Schematic drawing of (B) MSV-NFM gene; (C) MSV-NFH gene; and (D) MSV-LLH gene. The introns in C are not drawn to scale and the actual size of introns are indicated. (D) Shown at the bottom of D is a schematic drawing of MSV-LLH polypeptide. The encoded amino acid numbers are shown below the arrowheads. The numbers in the parentheses represent the amino acid numbering from the wild-type NF-H polypeptide for the NF-H tail segment in LLH.

all three NF subunits indicates that each may be an integral subunit of the 10-nm NFs. Assembly studies using purified NF-subunits show that only NF-L and NF-M, but not NF-H, are able in vitro to form 10-nm filaments (Gardner et al., 1984; Geisler and Weber, 1981; Liem and Hutchinson, 1982), although even under optimal conditions NF-M self-assembles into short, irregular filaments and only under very limited buffer conditions. Both NF-M and NF-H can be incorporated into filaments in the presence of NF-L, indicating that determinants for coassembly are contained within the individual subunits (Balin and Lee, 1991; Hisanaga and Hirokawa, 1988; Zackroff et al., 1982). These in vitro assembly experiments, along with antibody decoration of native neurofilaments (Hirokawa et al., 1984; Willard and Simon, 1981), have led to the conclusion that in vivo neurofilaments are probably assembled from an NF-L core and that NF-L is necessary and sufficient to form filaments. Thus, potential functional roles for both NF-M and NF-H have focused on interactions mediated by the long carboxy-terminal tail domains (438 and 676 amino acids for NF-M and NF-H, respectively) which are targets for multiple phosphorylation events (see Nixon and Shea, 1992). However, interpretation of neurofilament reassembly studies is not as straightforward as it may seem. In vitro reassembly of neurofilaments requires acidic pH, is inefficient when compared to the reassembly of either Type III filaments (vimentin) or Type I/II filaments (keratin) and yields short, somewhat irregular filaments. Moreover, neurofilaments reassembled in vitro do not approximate the parallel arrays of organized neurofilaments seen in all regions of a neuron.

In vivo assembly characteristics of NF subunits have also been studied by expressing NF subunits in fibroblast cells in culture and examining the coassembly of NF subunits with endogenous vimentin filaments (Monteiro and Cleveland, 1990; Gill et al., 1990; Wong et al., 1990; Chin and Liem, 1990; Chin et al., 1991). Using such pseudo-in vivo systems, the rod as well as portions of the head and tail domains of both NF-L and NF-M have been shown to be important for coassembly with vimentin into a cytoplasmic network of filaments. We have now extended such analyses by examining the self-assembly and organization of NF subunits into a cytoplasmic neurofilament network in cells that do not express any cytoplasmic intermediate filaments. What we find is that the de novo assembly of neurofilaments in vivo requires both NF-L and either NF-M or NF-H. Further, we demonstrate that the amino-terminal head, but not the carboxyl-terminal tail, of NF subunits are required for assembly of a neurofilament network in vivo.

Materials and Methods

Construction of Hybrid NF Genes

The construction of murine sarcoma virus (MSV) promoted NF genes, as well as amino and carboxyl-terminal truncation mutants, have been described (Monteiro and Cleveland, 1990; Gill et al., 1990; Wong and Cleveland, 1990). Cytomegalovirus-promoted vimentin (pCMV-vimentin) (Sommer et al., 1992) was a kind gift from Dr. Connie Sommers (National Institutes of Health, Bethesda, MD).

To construct pCMV-NFL (Fig. 1 A), pNF-L-Bam (Monteiro and Cleveland, 1990) was linearized at the BamHI site located between the promoter and the ATG translation initiation codon and blunted with Klenow and dNTP. The DNA was then digested with HindIII and the 5-kb NF-L gene fragment missing the 5'-promoter region was isolated. To produce pCMV, the 600-bp human CMV early promoter was cloned between KpnI and XhoI sites in pGEM-3z. To produce pCMV-NF-L, pCMV was digested with XhoI, blunted with Klenow and digested with HindIII. The 5-kb 5'-blunt/3'-HindIII NF-L fragment from above was then ligated into blunt/HindIII sites 3' to the CMV promoter.

To construct pMSV-NFH (Fig. 1 C), a 12-kb fragment containing the mouse NF-H gene was obtained by double digesting Cos3Al (kindly provided by Dr. J.-P. Julien, Montreal, Canada; Julien et al., 1988) with KpnI and SalI. To obtain pNFH, the 12-kb NF-H gene was cloned between KpnI and SalI sites of pUC19. To obtain pMSV-NF-H, the NF-H promoter in pNFH was removed using KpnI and NotI and replaced with the MSV promoter described below. A 600 base (HindIII-BglII fragment) containing the MSV promoter was subcloned between the HindIII-BamHI sites of pMSV-NF-L to generate pMSV-NF-H. Oligonucleotide-directed mutagenesis was used to create HindIII restriction sites in both NF-L and NF-H genes and at exactly the same position in the carboxy-terminal portion of the rod, which ends in a sequence (RKLEGE) common to both NF-L and NF-H. The nucleotide changes did not alter the amino acid sequence (Fig. 1 D). For NF-H, a 2.3-kb EcoRI fragment (encoding amino acids 267-1053 of NF-H) from a partial NF-H cDNA clone, pMuHII (kindly provided by Dr. P. Shneidman, University of Pennsylvania; Shneidman et al., 1988), was subcloned into the EcoRI site of M13mpl8. Single-stranded DNA was obtained from a clone with desired orientation and a HindIII site was engineered into the gene by oligonucleotide-directed mutagenesis using the Amersham kit, version 2.1 (Amersham Corp., Arlington Heights, IL). The mutants were confirmed by DNA sequencing. Double-stranded DNA was recovered and digested with HindIII and EcoRV. The mutated 1.3-kb fragment was cloned between the EcoRI-HindIII sites of pBSII-KS* to produce pMuHII(H3)-BS. To introduce a HindIII site in the NF-L gene, 1 kb KpnI fragment containing the sequences encoding the terminal rod domain (including the KLLEGE) was subcloned into the KpnI site of pBSII-KS*. Single stranded DNA templates were rescued (using helper phage R408)
Individual NF subunits are self-assembly incompetent in an in vivo context. IF- SW13 cells were grown on glass coverslips and transfected with either (A) pMSV-NFH, (B) pMSV-NFM, (C) pMSV-NFL, or (D) pCMV-Vim. Using immunofluorescence microscopy, (A) NF-H and (C) NF-L were localized by affinity-purified rabbit polyclonal antibodies to either NF-L or NF-H followed by fluorescein-conjugated goat anti-rabbit IgG. (B) NF-M and (D) vimentin were localized by mouse anti-NF-M or anti-vimentin mAbs followed by fluorescein-conjugated horse anti-mouse IgG. Bar, 10 μm.

Figure 2. Individual NF subunits are self-assembly incompetent in an in vivo context. IF- SW13 cells were grown on glass coverslips and transfected with either (A) pMSV-NFH, (B) pMSV-NFM, (C) pMSV-NFL, or (D) pCMV-Vim. Using immunofluorescence microscopy, (A) NF-H and (C) NF-L were localized by affinity-purified rabbit polyclonal antibodies to either NF-L or NF-H followed by fluorescein-conjugated goat anti-rabbit IgG. (B) NF-M and (D) vimentin were localized by mouse anti-NF-M or anti-vimentin mAbs followed by fluorescein-conjugated horse anti-mouse IgG. Bar, 10 μm.

from a clone with the desired insert orientation and a HindIII site was introduced by oligonucleotide-directed mutagenesis. The desired clone, pNFL(Kpn-H3), was identified using both restriction digestion with HindIII and DNA sequencing. pNFL(Kpn-H3) was then digested with Kpnl and HindIII to obtain a 450-bp fragment containing the sequences just 5' to the KLEGGE-domain of NF-L. This fragment was ligated 5' to pMuHI(H3)-BS, which had been linearized by double digesting with Kpnl and HindIII. The resulting clone, pl(Kpn)(Hi(H3))-BS, contains the 3' half of NF-L exon 2, intron 3 and the engineered HindIII site located 1 base into exon 3, followed by the NF-L tail region up to sequences encoding amino acid 798. To complete the carboxyl-terminal region of NF-H and to add a myc-epitope tag, an 870 base fragment beginning at codon 799 and ending at the normal translation termination codon was obtained by PCR. The PCR primers were designed so that the last four amino acids and the termination codon of NF-H were replaced with a Ncol site followed by a 12-amino acid myc-tag. The PCR fragment was digested with Ncol and ligated into pmyc-0 (a plasmid carrying the myc-tag followed by the NF-L 3' gene flanking sequences; Wong and Cleveland, 1990) that had been first digested with Sall, blunted by Klenow and digested with Ncol. The resulting clone (pNFHc284-myc) contains the NF-H sequences encoding the last 284 amino acids of NF-H tail followed by the pmyc-0 sequences. From pNFHc284-myc, a 1.4-kb fragment encoding 284 amino acids of NF-H, the myc-tag and the NF-L poly A-signal was excised by double digesting with EcoRV and EcoRI. To obtain pH(Kpn-H3)Ht, this 1.4-kb fragment was ligated to pL(Kpn)HI(H3)-BS that had been linearized by double digesting with EcoRV and EcoRI.

pMSV-LLH was built from three parts. To obtain the first of these, pMSV-NFL (Monteiro and Cleveland, 1990) was double digested with BgIII and EcoRI, yielding the pUC vector plus the MSV promoter, NF-L sequences in exon 1 up to a BgIII site, and the NF-L 3' flanking region. For the second part, the NF-L gene was double digested with BgIII and Kpnl to obtain a 1.8-kb fragment containing the 3'-end of NF-L exon 1, NF-L intron 1 and 3' half of exon 2. For the third fragment, pl(Kpn-H3)Ht was double digested with Kpnl and EcoRI; the resulting 3-kb fragment contained the NF-sequences and the myc-tag was purified. These fragments were ligated together to obtain pMSV-LLH.

Tissue Culture and Transfection

Vimentin containing (cl.1; IF+) and vimentin negative (cl.2; IF-) clones of the SW13 cell line (derived from a human adrenal carcinoma (Sarria et al., 1990) were kindly provided by Dr. Robert M. Evans (Department of Pathology, University of Colorado Health Sciences Center). The SW13 cells were grown in DME supplemented with 10% FBS. The SW13 cells were transiently transfected using the calcium phosphate co-precipitation procedure (Graham and van der Eb, 1973). The cells were routinely analyzed 40 h after transfection.

Mouse fibroblast L-cells were maintained in DME supplemented with 10% FBS. The L-cells were transiently transfected using the DEAE-dextran method (Lopata et al., 1984). Cells were analyzed 40 h after transfection.
Immunofluorescent Staining and Light Microscopy

Cells grown on glass coverslips were washed in microtubule stabilization buffer (MSB: 4 M Glycerol, 100 mM Pipes-KOH, pH 6.9, 1 mM EGTA and 1 mM MgSO4) for 30 s at 35°C, extracted for 60 s in MSB containing 0.4% (wt/vol) Triton X-100 at 35°C and briefly rinsed in MSB. The samples were then fixed by immersion in ~20°C methanol for 10 min. The samples were rehydrated in PBS, blocked with 2% nonfat dry milk for 15 min at room temperature, washed in PBS and incubated with a primary antibody (diluted in PBS containing 0.2% nonfat dry milk) for 1-2 h at room temperature or overnight at 4°C. After the primary antibody incubation, coverslips were washed three times in PBS, incubated for 1 h in secondary antibody, washed again in PBS, and mounted on glass slides using Aquamount (Lerner Laboratories, New Haven, CT). The cells were examined on an Olympus BH-2 microscope using epifluorescence optics and photographed on Kodak T-MAX film (Eastman Kodak Co., Rochester, NY). Proteins containing the 12-amino acid myc epitope tag were detected using either an anti-myc mouse mAb (9E10; Evan et al., 1985) or affinity-purified rabbit polyclonal antibodies generated against a myc synthetic peptide (CYG-MEQKLISEEDLN). Specific NF subunits were also detected using mouse mAbs to either NF-L, NF-M, or NF-H, and affinity-purified rabbit polyclonal antibodies to NF-L or NF-H (Xu et al., 1993). A goat anti-vimentin polyclonal antibody (ICN Immunobio-logicals, Costa Mesa, CA) and an anti-vimentin mouse mAb (YB; Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN) were used to detect vimentin. Appropriate affinity-purified secondary antibodies conjugated to either Texas red or fluorescein were used.

SDS Gel Electrophoresis and Immuno blotting

Cells were usually harvested 40 h after transfection, washed once with PBS, once with MSB at 35°C, extracted for 1 min with MSB containing 0.4% Triton X-100 at 35°C, and washed once with MSB at 35°C. The remaining cytoskeletal fraction was solubilized in 2% (wt/vol) SDS in 50 mM Tris-HCl, pH 6.8. Extracts were boiled for 5 min (100°C) and protein concentrations were determined using the bicinchoninic acid method (Smith et al., 1985). Extracts were then diluted with SDS sample buffer containing β-mercaptoethanol, boiled again for 5 min, and equal amounts of cytoskeletal proteins were separated by electrophoresis on 7.5% polyacrylamide gels (Laemmli, 1970). The separated proteins were immunoblotted (Lopata and Cleveland, 1987) and NF-L, NF-M, NF-H and vimentin were detected by autoradiography using mouse monoclonal primary antibodies against the appropriate proteins and 125I-labeled goat anti-mouse secondary antibody. To quantify the amount of NF subunits accumulated in any sample, a series of twofold dilutions of neurofilament purified from mouse spinal cord and containing known amounts of each NF subunit were immunoblotted in parallel with the samples of interest. The immunoreactivity of specific NF-subunit bands was quantified by densitometric scanning of the autoradiogram and compared with a standard curve constructed from densitometric scan of the neurofilament standards.

Ultrastuctural Analysis of Transgenic Mice

Characterization of transgenic mouse lines expressing wild-type NF-L under the control of a MSV promoter (MSV-NFL-58 and MSV-NFL-103) has been previously described (Xu et al., 1993; Monteiro et al., 1990). Similar transgenic animals expressing a NF-M polypeptide in which the last 50 carboxyl-terminal amino acids were replaced by a 12-amino acid myc epitope tag (MSV-NF-Mca50; Wong et al., 1990) were generated by standard transgenic methods. A detailed characterization of these mice will be described elsewhere (Wong, P. C. J., Marszalek, and D. W. Cleveland, manuscript in preparation). Transgenic mice expressing both NF-L and NF-Mca50 in oligodendrocytes were engineered by mating MSV-NFL-58 with mice expressing MSV-NF-Mca50.

Mouse tissues were fixed for immunocytochemistry by intracardial perfusion with either 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.6. For EM, tissues were fixed by intracardial perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate, pH 7.6. For immunocytochemistry, the spinal cords were cryoprotected in 20% glycerol in 0.1 M sodium phosphate, pH 7.6 and frozen sections were cut (40 µm). The transgenic NF subunits in nonneuronal cells in the spinal cord white matter were localized using double immunofluorescence microscopy. NF-L and NF-Mca50 were colocalized using affinity-purified anti-NF-L rabbit polyclonal antibodies (Xu et al., 1993) and anti-myc mouse mAb (9E10; Evan et al., 1985) as primary antibodies. Astrocytes were identified using a rabbit polyclonal antibody to GFAP (Dako Corp., Carpenteria, CA).

For EM, paraformaldehyde/glutaraldehyde fixed spinal cords were post-fixed with 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in alcohol and embedded in Epon-Araldite resin. Thin sections were cut and stained with uranyl acetate and lead citrate and examined in an Hitachi H-600 transmission electron microscope.

Results

Individual Neurofilament Subunits Are Self-assembly In competent In Vivo

To examine the ability of each NF subunit to self-assemble an extended, de novo filament network in vivo, wild-type mouse NF-L, NF-M, and NF-H were individually expressed in IF- SW13 cells, a cloned subline in which vimentin expression has been spontaneously silenced (Sarra et al., 1990). NF subunits and vimentin were localized 40 h after transfection using double immunofluorescence microscopy. (Simultaneous localization of vimentin and NF subunits was routinely done to control for the small percentage of the cells [~1-5%] that spontaneously revert to IF+ phenotype.) As expected from its known in vitro assembly properties, in the absence of an endogenous IF array, NF-H failed to assemble into a filament network and localized to nonfilamentous, punctate structures that extended out to the peripheral margins of the cell (Fig. 2 A). When expressed alone, NF-M was also incompetent to self-assemble within an in vivo context and localized to nonfilamentous structures similar to those seen with NF-H (Fig. 2 B). In view of its assembly competence in vitro, expression of NF-L in IF- cells produced an even more surprising result. At lower levels of accumulation, NF-L was restricted to fine punctate structures (not shown) similar to those seen with NF-M and NF-H. At higher levels of NF-L (achieved with the CMV-NFL gene), NF-L formed large nonfilamentous aggregates that covered large portions of the cytoplasm (Fig. 2 C).

The absence of an IF network assembled from individual neurofilament subunits does not represent an inherent defect in IF assembly in the SW13 IF- line: forced expression of human vimentin yielded a well elaborated vimentin filament network in the same cells (Fig. 2 D; also see Sarria et al., 1990). Quantitative immunoblotting to measure average levels of accumulated NF-L, NF-M, NF-H, or vimentin showed that failure to assemble networks from the NF subunits could not be explained by low levels of expression (see below).

Figure 3. Coassembly of individual NF subunits with endogenous vimentin in IF+ SW13 cells. SW13-c1.1 cells were grown on glass coverslips and transfected with plasmid (A and B) pCMV-NFL, (C and D) pMSV-NF-M, (E and F) pMSV-NF-N. Mouse L-cells grown on glass coverslips were transfected with (G and H) pMSV-NF-H. (A, C, and G) NF subunits were localized using affinity-purified rabbit polyclonal antibodies against (A) NF-L or (E) NF-H or mouse mAbs against (C) NF-M and (G) NF-H. Appropriate fluorescein-conjugated secondary antibodies were used to visualize expression by indirect immunofluorescence microscopy. (B, D, F, and H) Vimentin in the same transfected cells was visualized using double immunofluorescence microscopy with (B and F) mouse mAbs or (D and H) goat polyclonal antibodies to vimentin followed by appropriate Texas red–conjugated secondary antibodies. Bar: (A-F) 10 µm; (G and H) 16 µm.
Further, the mouse NF-L and NF-M subunits were fully competent for coassembly with human vimentin in IF+ SW13 cells (Fig. 3, A-D), results consistent with earlier transfection experiments in other cells (Monteiro and Cleveland, 1990; Chin et al., 1991). Similarly, in cells expressing low levels of NF-H (i.e., those with dimmer fluorescent signals) NF-H too coaligned into a vimentin filament network (as in other cells; Chin and Liem, 1990). However, as might be expected from its in vitro behavior, higher levels of the completely wild type NF-H polypeptide yielded nonfilamentous, punctate aggregates and a partially disrupted the endogenous vimentin network both in SW13 IF+ [Fig. 3, E and F] and mouse L cells [Fig. 3, G and H].

We conclude that, since no single NF subunit is capable of forming a homopolymeric filament network in the IF-SW13 cell, this must reflect a network assembly defect in each individual subunit.

**NF-M or NF-H Complements In Vivo Network Assembly Incompetence of NF-L**

Since NF-L in vivo is always expressed in conjunction with other IF subunits (see Nixon and Shea, 1992), the fact that NF-L alone is not sufficient to form an in vivo filament network suggests that multiple NF subunits may be required to form a neurofilament network in vivo. To examine directly the possibility that neurofilaments in vivo are obligate heteropolymers, co-transfection was used to express NF-L with NF-M or NF-H in IF+ SW13 cells. In co-transfected cells, NF-L always assembled into a distinctive filamentous network consisting of long individual filaments, as well as thick bundles of filaments (Fig. 4). In extreme cases, lateral association of filaments yielded thick cables of filaments containing both NF-L (cell at lower right in Fig. 4 A) and NF-M (Fig. 4 B). In all cases, both NF-M (Fig. 4 B) and NF-H

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**Figure 4.** Coexpression of NF-L with either NF-M or NF-H drives de novo assembly of neurofilaments in IF+ SW13 cells. SW13 cells were co-transfected with pCMV-NFL and either (A and B) pMSV-NFM or (C and D) pMSV-NFH. (A and C) Immunofluorescence localization of NF-L by affinity-purified rabbit anti-NF-L antibodies followed by fluorescein-conjugated secondary IgG. (B) NF-M and (D) NF-H were colocalized with NF-L in the same NF-L transfected cells shown in A and B using double immunofluorescence microscopy by mouse monoclonal anti-NF-M or NF-H antibodies followed by Texas red-conjugated horse anti-mouse IgG secondary. Bar: (A and B) 16 μm; (C and D) 10 μm.
Sequences within the Amino-terminal Head, but Not the Carboxyl-terminal Tail, of NF-M and NF-L Are Essential for De Novo Assembly of Neurofilaments In Vivo

To examine what domain(s) of NF-M is necessary for complementation of de novo assembly of NF-L into a filament network in vivo, a series of carboxyl-terminal or amino-terminal truncation mutants of NF-M were co-transfected into the entire tail domain of NF-M was expressed with wild-type NF-L, the mutant NF-M coassembled with NF-L and drove the assembly of a de novo neurofilament network. Even truncation of the entire tail (NF-M-CA438, Fig. 6 E) still complemented network assembly from NF-L. Further, among the different NF-M mutants and the wild-type NF-M, there were no consistent differences in the actual morphology of the assembled networks. The images shown (Fig. 6, A–F) give representative examples of various morphologies of networks that are observed with all NF-M subunits. In particular, filament bundling into thick cables is a prominent feature. Further truncation into the rod domain of NF-M (e.g., NF-M-CA443, not shown) did not support NF-L assembly. These results demonstrate that the entire tail domain of NF-M is dispensable for driving network assembly of NF-L in an in vivo context.

Next, the necessity of the amino-terminal head region of NF-M for de novo filament assembly with NF-L was examined. When NF-L was expressed with NF-M deleted 42 amino acids from its amino terminus (NF-M-Nzx42), NF-M-Nzx42 was able to coassemble with NF-L and drive the assembly of a filament network in complete absence of vimentin (Fig. 6, G and H). However, when NF-M missing 75 amino-terminal amino acids (NF-M-Nzx75) was expressed with the wild-type NF-L, both NF-M-Nzx75 and NF-L colocalized to nonfilamentous, punctate aggregates (Fig. 6, I and J). Thus, amino acid sequences contained within the region between Ser42 and Ser75 in NF-M are essential for.
The roles of the NF-L amino-terminal head and carboxyl-terminal tail in de novo assembly of neurofilaments were also examined. To test if any specific sequence within the entire carboxyl-terminal tail region of NF-L is essential for assembly of a filament network in vivo, a hybrid NF-gene was constructed to encode a subunit in which the carboxyl-terminal tail of NF-L was replaced with the entire carboxyl-terminal tail of NF-H (Fig. 1D). The hybrid protein, named LLH (the letters denote the subunit origin of the head, rod and tail, respectively) coassembles with the endogenous vimentin into cytoplasmic filament networks without any obvious signs of filament disruption or formation of nonfilamentous aggregates (Fig. 7, A and B). Thus, the assembly properties of the LLH hybrid in the presence of vimentin better approximates NF-L than NF-H, since at comparable levels of expression NF-H disrupts filament networks in a significant number of cells. In IF- SW13 cells, the expression of the LLH hybrid alone (not shown) or co-expression with wild-type NF-L (Fig. 8, A and B) results in localization of the LLH hybrid into nonfilamentous aggregates without any obvious signs of filament disruption or formation of nonfilamentous aggregates (Fig. 7, A and B). Thus, the assembly properties of the LLH hybrid in the presence of vimentin better approximates NF-L than NF-H, since at comparable levels of expression NF-H disrupts filament networks in a significant number of cells. In IF- SW13 cells, the expression of the LLH hybrid alone (not shown) or co-expression with wild-type NF-L (Fig. 8, A and B) results in localization of the LLH hybrid into nonfilamentous aggregates without any obvious signs of filament disruption or formation of nonfilamentous aggregates (Fig. 7, A and B). Thus, the assembly properties of the LLH hybrid in the presence of vimentin better approximates NF-L than NF-H, since at comparable levels of expression NF-H disrupts filament networks in a significant number of cells.

Figure 6. Sequences within the amino-terminal head but not carboxyl-terminal tail of NF-M are essential for complementing de novo assembly of NF-L in vivo. IF- SW13 cells were co-transfected with pCMV-NFL and a series of either (A-F) carboxyl-terminal or (G-J) amino-terminal truncation mutants of NF-M. Numbers indicate deletion endpoints from either amino or carboxyl termini and the approximate borders of deletions are shown on the schematic of NF-M protein at the top. All of the NF-M mutant proteins contain a 12-amino acid myc-tag at the extreme carboxyl-terminus. (A, C, E, G, and I) Mutant NF-M subunits were localized by immunofluorescence microscopy using mouse monoclonal anti-myc-tag antibodies followed by fluorescein-conjugated horse anti-mouse IgG secondary. (B, D, F, H, and J) NF-L subunits in same transfected cells as in A, C, E, G, and I were localized by double immunofluorescence microscopy using affinity-purified rabbit anti-NF-L antibody followed by Texas red-conjugated goat anti-rabbit IgG secondary. Bar, 10 μm.
**Figure 8.** LLH complements de novo assembly incompetence of NF-M. IF\(^-\) SW13 cells were co-transfected with pMSV-LLH and either (A and B) pMSV-NFL or (C and D) pMSV-NF-M. (A and C) The LLH-hybrid was localized by affinity-purified rabbit anti-myc polyclonal antibodies followed by fluorescein-conjugated secondary. Double immunofluorescence microscopy was used to localize (B) NF-L and (D) NF-M by mouse mAbs to either NF-L or NF-M, followed by Texas red-conjugated secondary. Bar, 10 \(\mu\)m.

**Assembly of Neurofilament Networks in IF\(^-\) Oligodendrocytes of Transgenic Mice Expressing Both NF-L and NF-M, but Not Either Subunit Alone**

To examine the de novo assembly properties of NF-L and NF-M in another in vivo context, we exploited two sets of transgenic mice that express MSV-promoted transgenes encoding either wild-type mouse NF-L (Monteiro et al., 1991; Xu et al., 1993) or a mouse NF-M subunit in which the last 50 carboxy-terminal tail amino acids were replaced with a 12-amino acid epitope tag (Wong, P. C., J. Marszalek, and D. W. Cleveland, manuscript in preparation). Unlike the endogenous NF-L and NF-M genes, immunocytochemistry of frozen sections of tissues from transgenic animals revealed that both transgenes are expressed in some non-neuronal cells, including in oligodendrocytes of the lumbar spinal cord (data not shown). Since these cells, which myelinate central nervous system neurons, do not normally express cytoplasmic intermediate filaments (e.g., Peters et al., 1991), we used EM of tissue sections of NF-L or NF-M transgenic animals to examine de novo filament network assembly. Just as in transfected SW13\(^+\) cells, extended filament arrays were not observable in oligodendrocytes of heterozygotes of either transgenic line (Fig. 10, A and B). However, examination of oligodendrocytes produced by mating these two lines revealed that expression of both NF-L and NF-M yielded prominent, closely spaced bundles of 8-10-nm filaments (Fig. 10 C) that, at this level of resolution, were essentially indistinguishable from those found in neurons. Since such filament arrays were not found in oligodendrocytes from animals homozygous for either transgene, filament assembly in the NF-L/NF-M doubly transgenic animals cannot be due simply to increased expression of NF polypeptides; rather, it must result from assembly promoting interactions between NF-L and NF-M subunits.

**Discussion**

By transfection into a IF\(^-\) cultured cell line and by forcing expression of NF subunits in IF\(^-\) oligodendrocytes in transgenic mice, we have uncovered the unexpected finding that, despite self assembly in vitro, NF-L is not sufficient for assembly of an NF network. Defective by itself, network assembly is restored to NF-L by substoichiometric amounts of NF-M or NF-H, neither of which alone or in combination with each other assemble networks. The obvious conclusion is that in vivo neurofilaments are obligate heteropolymers assembled from NF-L and NF-M/NF-H. Since tailless NF-M also complements NF-L assembly, but truncations into the amino-terminal head cannot, sequences in the NF-M head (and rod) provide the essential interaction for network formation by NF-L. Although the requirement of the NF-H head has not been directly tested, its tail (carried by hybrid subunit LLH) does not complement NF-L assembly. Thus, it seems highly likely that for NF-H, too, sequences in the head (and rod) mediate in vivo NF-L assembly.
Figure 9. The amino-terminal head of NF-L is essential for de novo coassembly with NF-M. IF- SW13 cells were co-transfected with pMSV-NFM and either (A and B) pMSV-NFL-N_{22} or (C and D) pMSV-NFL-N_{89}. Immunofluorescence microscopy was used to localize (A) NFL-N_{22} or (C) NFL-N_{89} by affinity-purified rabbit polyclonal antibody followed by fluorescein-conjugated secondary. (B and D) Double immunofluorescence microscopy was used to localize NF-M by mouse monoclonal anti-NF-M antibodies, followed by a Texas red-conjugated secondary. Bar, 10 μm.

The molecular basis for inability of NF-M and NF-H to form heteropolymeric filaments together in vivo is not clear. However, it is reasonable to assume that the carboxyl-terminal tail domains of these subunits do not inhibit the actual assembly process, as shown by LLH/NFM heteropolymeric filament assembly (Fig. 8). Possibly the rod domains of NF-M and NF-H do not interact in a productive manner and may need to interact with NF-L. The structural basis for this possibility is suggested by the fact that unlike the assembly competent type I, II, III, and other IV subunits, neither NF-M nor NF-H rods has the characteristic disruption in the \( \alpha \)-helix in coil I (Lees et al., 1988; Myers et al., 1987). However, nothing in the present evidence excludes the alternative that some domain in the NF-L amino-terminal head may also be required for proper coassembly.

That neurofilaments in vivo are obligate heteropolymers requiring NF-L and either NF-M or NF-H clearly establishes the functional importance of NF-M and NF-H in the assembly and organization of neurofilaments. An obvious extension of this finding is that the assembly of neurofilaments in neurons may be affected by posttranslational regulation of any one of the NF subunits. Both the obligate heteropolymeric nature and the tendency for these filaments to bundle laterally in vivo resemble the properties of keratin filaments (Bader et al., 1991; Lu and Lane, 1990). However, the assembly of neurofilaments and keratins differ in one important area: the keratin filaments are stoichiometric heterodimers requiring one Type I and Type II keratin. In contrast, neurofilaments can apparently accommodate a wide range of NF-subunit ratios, a point of some significance since the NF-
Figure 10. De novo assembly of an NF network in oligodendrocytes of transgenic mice requires expression of both NF-L and NF-M transgenes. Thin section electron micrographs of oligodendrocytes expressing (A) NF-L alone, (B) NF-McA50 alone, and (C) both NF-L and NF-Mczx50. The insets show the cells from which A, B, and C were taken. Bar: 750 nm; (insets) 6 μm.

Subunit ratios do change dramatically during neuronal development (see Nixon and Shea, 1992). Whether the requirement for heteropolymerization acts at the dimer, tetramer or higher order oligomer level has not yet been determined, although crosslinking experiments (Carden and Eagles, 1986) and antibody labeling of filaments assembled in vitro (Mulligan et al., 1991) are most easily explained by NF-L/NF-M or NF-L/NF-H heterodimers.

Another feature that was not revealed by in vitro reassembly studies or previous transfection studies is the propensity for neurofilaments assembled in vivo to interact laterally to form bundles. Although some lateral interaction of filaments is seen with vimentin alone (Fig. 2 D) or when NF-subunits are coexpressed with vimentin (Fig. 3, A and C), the bundles are neither as striking or abundant as that observed for NF assembled in the absence of vimentin (e.g., compare Fig. 3, A and C with Fig. 4, A, C, and E). Possibly this is because vimentin (and other Type III subunits) contain a region within the carboxyl-terminal domain that prevents filaments from laterally associating (Kouklis et al., 1991). On the other hand, neurofilaments may possess an inherent tendency to interact laterally, thereby allowing the carboxyl-terminal tail regions of NF-M and NF-H to modulate lateral spacing between filaments by steric and electrostatic interactions. This hypothesis is consistent with the observation that the carboxyl-terminal tail region of both NF-M and NF-H extend from the surface of the filaments (Hisanaga and Hirokawa, 1988). However, if this is true, what remains unclear is why neurofilaments have little lateral affinity in vitro.

While the carboxyl-terminal tails are likely to be involved in regulation of higher order interaction between filaments and other cellular structures, the tails are not likely to be involved directly in actual assembly of neurofilaments in vivo. Even a completely tailless NF-M stimulates network coassembly with NF-L. At first glance, the efficient assembly of tailless NF-M (NFM-Ca438) with NF-L into a filament network may seem at odds with a previous report that this truncated NF-M is a dominant assembly disrupter (Wong and Cleveland, 1990). However, this is probably related to the differences in the level of expression after transfection into different cells; higher levels disrupt neurofilament arrays. Although NFL-Nα89 has also been reported to be an assembly defective mutant (Gill et al., 1990), differences in the levels of mutant accumulation also probably account for coassembly of this “headless” NF-M mutant with the wild-type vimentin array observed here and by others (Chin and Liem, 1991). Overall, the current results are consistent with effects of carboxyl-terminal tail mutations on other filament types which collectively show that the amino-terminal head region is much more directly involved in filament assembly than are the carboxyl-terminal tail domains (Raats et al., 1992, 1991, 1990; Bader et al., 1991; Lu and Lane, 1990; Kaufmann et al., 1985; also see Stewart, 1993). One question that remains untested is whether a filament network can be assembled from tailless NF-L and NF-M. For other IF subunits, several studies collectively suggest that filament assembly with tailless mutants may require presence of wild-type subunits (Albers and Fuchs, 1987; Lu and Lane, 1990; Raats et al., 1991; Eckelt et al., 1992; also see Coulombe, 1993).

The importance of the amino-terminal head region in assembly of neurofilaments in vivo has particular relevance to the regulation of neurofilament assembly in vivo. Direct involvement of amino-terminal head region of IF-subunits in filament assembly is indicated by deletion mutagenesis experiments on many IF subunits (Raats et al., 1992, 1991, 1990; Bader et al., 1991; Lu and Lane, 1990; Kaufmann et al., 1985; also see Stewart, 1993), including neurofilaments (Gill et al., 1990; Wong et al., 1990; Chin et al., 1991). The importance of the head domains for neurofilament assembly is further underscored by our finding that truncation of 75% of the NF-M head (NFM-NΔ75) fails to complement NF-L assembly, although it can coassemble with self-assembly competent vimentin (Wong and Cleveland, 1990). Further, the amino-terminal head domain of Type III, IV, and V subunit ratios do change dramatically during neuronal development (see Nixon and Shea, 1992). Whether the requirement for heteropolymerization acts at the dimer, tetramer or higher order oligomer level has not yet been determined, although crosslinking experiments (Carden and Eagles, 1986) and antibody labeling of filaments assembled in vitro (Mulligan et al., 1991) are most easily explained by NF-L/NF-M or NF-L/NF-H heterodimers.
subunits are known to be substrates for phosphorylation in vivo and phosphorylation of this region either inhibits assembly or causes disassembly of filaments both in vitro and in vivo (Ando et al., 1989; Evans, 1989; Inagaki et al., 1989; Kitamura et al., 1989; Chou et al., 1990; Hisanaga et al., 1990; Peter et al., 1990; Sihag and Nixon, 1990, 1991). For the NF subunits, major in vivo phosphorylation sites on the amino-terminal head domain are either located within (Ser27 on NF-L and Thr48 on NF-M; Sihag and Nixon, 1990, 1991) or are very near (a phosphopeptide starting at Ser25 on NF-M; Sihag and Nixon, 1990) the sequences essential for in vivo neurofilament assembly. Additionally, the phosphate on Ser55 of NF-L disrupts assembly. Collectively, it seems very likely that modification of the head domains on NF subunits affects assembly. Among the interesting possibilities for regulation include blocking premature assembly prior to transport into neurites. It remains for future efforts to define more clearly how each modification ultimately affects network assembly.

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