Neurofilaments (NFs) are major cytoskeletal components of neurons and are essential for establishing the correct diameters of large myelinated motor and sensory axons (Friede and Samorajski, 1970; Hoffman et al., 1987; Lee and Cleveland, 1994). In an adult neuron, NFs are composed mainly of three different polypeptide subunits: NF-L (low molecular weight, 68 kDa); NF-M (intermediate molecular weight, 160 kDa); and NF-H (high molecular weight, 200 kDa). They belong to type IV intermediate filaments (IFs), according to their amino acid sequence similarity and the intron-exon position in their genomic sequence. Like all IFs, NF proteins contain a relatively conserved segmented α-helical rod domain of approx. 310 amino acids flanked by a variable N-terminal head domain and C-terminal tail domain (Steinert and Roop, 1988). While the rod domain of NF-L is similar to that of type-III IFs (Lee and Cleveland, 1994) with an interrupted heptad repeat in coil 1, this domain in NF-M (Levy et al., 1987) and NF-H (Lee et al., 1988; Chin and Liem, 1990) is a continuous coil. NFs in large myelinated axons are composed of a parallel array of 10 nm filaments with frequent crossbridges between adjacent filaments were formed in the cytoplasm of Sf9 cells infected with the recombinant virus that co-expressed NF-L and NF-H. To explore the function of the C-terminal tail domain of NF-H, various deletion mutants lacking portions of the tail domain were constructed, and each of them was coexpressed with NF-L. The results show that the tail domain of NF-H is a structural component of crossbridges and is involved in parallel bundle formation of neurofilaments, as core filaments of the axon. The last 191 amino acids of the C-terminal tail domain of NF-H play a key role in crossbridge formation.

**Key words:** Cytoskeleton, Neurofilament, Neurofilament-H, Morphogenesis

**SUMMARY**

In order to study the role of NF-H in a neurofilament network formation in neurons, we coexpressed NF-H with neurofilament protein-L (NF-L) in Sf9 cells using the baculovirus expression system. Electron microscopy observations revealed that parallel arrays of 10 nm filaments with frequent crossbridges between adjacent filaments were formed in the cytoplasm of Sf9 cells infected with the recombinant virus that co-expressed NF-L and NF-H. To explore the function of the C-terminal tail domain of NF-H, various deletion mutants lacking portions of the tail domain were constructed, and each of them was coexpressed with NF-L. The results show that the tail domain of NF-H is a structural component of crossbridges and is involved in parallel bundle formation of neurofilaments, as core filaments of the axon. The last 191 amino acids of the C-terminal tail domain of NF-H play a key role in crossbridge formation.

**Key words:** Cytoskeleton, Neurofilament, Neurofilament-H, Morphogenesis

**INTRODUCTION**

 Neurofilaments (NFs) are major cytoskeletal components of neurons and are essential for establishing the correct diameters of large myelinated motor and sensory axons (Friede and Samorajski, 1970; Hoffman et al., 1987; Lee and Cleveland, 1994). In an adult neuron, NFs are composed mainly of three different polypeptide subunits: NF-L (low molecular weight, 68 kDa); NF-M (intermediate molecular weight, 160 kDa); and NF-H (high molecular weight, 200 kDa). They belong to type IV intermediate filaments (IFs), according to their amino acid sequence similarity and the intron-exon position in their genomic sequence. Like all IFs, NF proteins contain a relatively conserved segmented α-helical rod domain of approx. 310 amino acids flanked by a variable N-terminal head domain and C-terminal tail domain (Steinert and Roop, 1988). While the rod domain of NF-L is similar to that of type-III IFs (Lee and Cleveland, 1994) with an interrupted heptad repeat in coil 1, this domain in NF-M (Levy et al., 1987) and NF-H (Lee et al., 1988; Chin and Liem, 1990) is a continuous coil. NFs in large myelinated axons are composed of a parallel array of 10 nm filaments with frequent crossbridges either between NFs or between NFs and microtubules (MTs) or membranous organelles (Hirokawa, 1982; Hirokawa and Takeda, 1998). These crossbridges are considered to maintain the spacing between the respective filaments and to form bundles of filaments arranged parallel, similar to the projection domains of MAP2 and tau protein, which determine the distance between microtubules in dendrites and axons (Hirokawa et al., 1984; Chen et al., 1992). In vitro reconstitution studies have revealed that NF-L can be assembled to form 10 nm core filaments by themselves, and many thin sidearms are observed projecting from the core when NF-M or NF-H is added (Hisanaga and Hirokawa, 1988). NF-M can also assemble to form short 10 nm filaments; however, NF-H can form only stubby structures under optimal conditions (Liem and Hutchison, 1982; Hisanaga and Hirokawa, 1988). Immunocytochemical studies show that specific antibodies against NF-L decorate the core filaments uniformly, and that the anti-NF-M antibody decorates both the core filaments and crossbridges; in contrast, the anti-NF-H antibody that recognizes the C-terminal tail domain of NF-H primarily decorates the crossbridges between the neurofilaments (Hirokawa et al., 1984). It is thought that the head and rod domains of NF-L, NF-M and NF-H form the core filaments, while the C-terminal tail domains of NF-M and NF-H form the crossbridges between the neurofilaments in neurons. Transfection studies have shown that each NF subunit is capable of coassembling with endogenous vimentin to form a 10 nm filament network (Chin and Liem, 1989; Chin and Liem, 1990; Monterio and Cleveland, 1989). However, despite the ability of NF-L to self-assemble into 10 nm filaments in vitro, neurofilament triplet proteins have been found to be incapable of homopolymeric assembly into a filamentous array in vivo, and neurofilaments are obligate heteropolymers requiring NF-L and NF-M or NF-H for their formation (Lee et al., 1993; Ching and Liem, 1993). When NF-Ls are expressed alone in Sf9 cells lacking endogenous intermediate filaments, they self-assemble into 10 nm short filaments, while NF-M aggregates in the cytoplasm in a detergent-insoluble form. Co-expression...
of NF-L and NF-M in Sf9 cells leads to their coassembly into bundles of 10 nm filaments packed in a parallel manner with numerous crossbridges resembling the neurofilament domains in axons. Thus, NF-M is thought to play a crucial role in a parallel-filament assembly. A series of deletion mutant experiments has shown that the C-terminal tail domain of NF-M is essential for crossbridge formation and longitudinal elongation of filaments (Nakagawa et al., 1995).

Neurofilaments fill most of the space in large myelinated axons. Disruption of the NF-L gene results in the absence of axonal neurofilaments, reduced radial growth of axons and delayed nerve regeneration (Zhu et al., 1997). This phenotype somewhat resembles the axons in a Japanese quail that carry a nonsense mutation of an NF-L subunit and lack neurofilaments in the nervous system (Yamasaki et al., 1991; Ohara et al., 1993), and those in mice overexpressing the NF-Hβ-galactosidase fusion protein in which the transport of neurofilament proteins into the axon is inhibited (Eyer and Peterson, 1994). The absence of NF-M caused by gene disruption is associated with decrease in the number of axonal calibers, expression levels of the NF-L subunit and neurofilament content, but increase in the expression levels of NF-H and number of microtubules. Thus, it is thought that NF-M is a major regulator of the expression level of NF-L and that its presence is required to achieve the maximal axonal diameter in all size classes of myelinated axons (Elder et al., 1998a). However, in NF-L-overexpressing transgenic mice, while the number of neurofilaments is increased two- to threefold, the axonal diameters are decreased slightly (Monterio et al., 1990; Xu et al., 1993). Increased NF-M expression is also associated with radial axonal growth and reduced expression levels of axonal NF-H, but it does not affect the nearest-neighbor spacing between neurofilaments (Wong et al., 1995). These results suggest that while neurofilaments are important for the radial growth of axons, the crossbridges between nearest-neighbors do not play a crucial role in radial axonal growth. Unlike the loss of the NF-L and NF-M subunits, loss of the NF-H subunit is associated with only a slight reduction in the nearest-neighbor spacing of neurofilaments, and it does not affect neurofilament distribution in either large- or small-diameter motor axons, but does result in increased microtubule density (Rao et al., 1998; Zhu et al., 1998; Elder et al., 1998b). Therefore, it is thought that the tail domain of NF-M governs the nearest-neighbor spacing of neurofilaments, and that NF-H is not required for establishing this spacing. Although results of immunocytochemistry suggested that NF-H is a component of crossbridges between NFs (Hirokawa et al., 1984), those of gene targeting studies fail to support this suggestion (Rao et al., 1998). Thus, the role of NF-H in crossbridge formation among NF domains remains unknown.

To directly examine the role of NF-H, particularly the C-terminal tail domain of NF-H, in crossbridge formation between adjacent NFs, and the cytoskeletal organization of neurons, we transfected cDNAs of NF-L and NF-H or their deletion mutants into insect Sf9 cells, which lack a cytoplasmic intermediate filamentous network (Nakagawa et al., 1995). The results showed that NF-H, particularly its C-terminal tail domain, has the ability to form crossbridges between adjacent neurofilaments and to stretch the neurofilaments to form parallel arrays of neurofilaments.
SDS-PAGE and immunoblot
The expression of neurofilament proteins and deletion mutants by each recombinant baculovirus was confirmed by SDS-PAGE and immunoblot analysis. Sf9 cells were infected with baculovirus encoding NF-H alone, both NF-L and NF-H, or each deletion mutant of NF-H. The samples from Sf9 cells infected with each baculovirus were processed by adding SDS-PAGE loading buffer to Sf9 cells that had been briefly washed with PBS. After gel electrophoresis, the proteins were transferred onto Immobilon™ membrane (Millipore) and probed with an anti-NF-L monoclonal antibody (NR4, Sigma), and with anti-NF-H monoclonal antibody (N52, Sigma), or with rabbit anti-NF-H polyclonal antibody (Chemicon). A peroxidase-conjugated anti-mouse IgG and peroxidase-conjugated anti-rabbit IgG (Cappel Laboratories) were used as secondary antibodies. The chromogenic reaction employed 4-chloro-l-naphthol.

Immunoelectron microscopy
Sf9 cells were washed briefly with PBS 40 hours after infection with LH virus. Cells were then permeabilized with 0.1% Triton X-100, 1 mM PMSF, 10 ng leupeptin in PEM buffer (100 mM Pipes, 1 mM EGTA, and 1 mM MgCl2, pH 6.8) for 10 minutes. Cells were harvested by centrifugation, and then resuspended in a fixative containing 2% paraformaldehyde, 0.1% glutaraldehyde and 0.1% Triton X-100 in PEM buffer for 30 minutes. After fixation, the cells were washed twice with PBS and quenched by 1 mg/ml NaBH4 in PBS for 20 minutes. They were then washed with PBS and incubated in PBS containing 5% BSA for 15 minutes. Monoclonal antibody against NF-L (NR4) and a polyclonal antibody against NF-H (Sigma) were used for LH-virus-infected cells. The cells were incubated with the primary antibodies at 37°C for 2 hours. After incubation, the cells were washed four times each with PBS for 10 minutes and with TBS (20 mM Tris-HCl, 150 mM NaCl2, pH 8.2) for 10 minutes. Blocking with the secondary antibody was carried out in TBS containing 5% BSA for 15 minutes. The cells were then incubated with colloidal-gold (10 nm)-conjugated goat anti-mouse IgG and gold (5 nm) -conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech) in the blocking solution at 4°C overnight, transferred to room temperature and further incubated for 2 hours. Thereafter, the cells were washed five times with TBS, for 30 minutes each. The cells were fixed with 2.5% glutaraldehyde for 1 hour at room temperature and postfixied with 1% OsO4 on ice. The samples were washed with distilled water. Cell pellets were dehydrated sequentially with ethanol and propylene oxide, and embedded in Epon. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined by electron microscopy (JEOL 1200 EX or 2010H).

Quick-freeze, deep-etch electron microscopy
Two days after infection, Sf9 cells were briefly washed in PBS and centrifuged at 700 rpm in a swing-out rotor for 3 minutes. The pellets were resuspended in PEM buffer containing 0.05% saponin and 2 mM GTP for 10 minutes at room temperature. Then the cells were harvested by centrifugation and fixed with 2.5% glutaraldehyde in the same solution as described above for 15 minutes at room temperature. Fixed cells were briefly washed with distilled water, quick-frozen with liquid helium and fractured as described previously (Hirokawa, 1982).

RESULTS
NF-H neither self-assembles nor aggregates in vivo
To examine the ability of NF-H subunits to self-assemble, we transfected NF-H alone, or both NF-L and NF-H together into Sf9 cells. Two different recombinant baculoviruses were produced. Results of western blotting showed that NF-H was expressed in H-virus-infected Sf9 cells, while both NF-L and NF-H were expressed in LH-virus-infected Sf9 cells (Fig. 2A). Monoclonal antibody N52 (which recognizes both phosphorylated and nonphosphorylated forms of NF-H) stained a band of NF-H expressed in Sf9 cells that corresponded to a molecular weight (180 kDa) higher than the molecular weight deduced from its amino acid sequence data (115 kDa), but lower than that of its phosphorylated form (200 kDa) in axons. The finding suggests that not all phosphorylation sites in the tail domain of NF-H are phosphorylated by kinases in Sf9 cells. The molecular weight of NF-L (68 kDa) is similar to that of the endogenous protein that was extracted from axons (Fig. 2A).

NF-H coassembles with NF-L and forms parallel 10 nm filament bundles with numerous crossbridges
We investigated the structures of expressed neurofilament proteins by electron microscopy. Sf9 cells infected with the H virus revealed a large amount of NF-H proteins accumulated in the cytoplasm below the cell membrane (Fig. 3A). Unlike NF-L and NF-M, which could assemble into short filaments or form tightly packed detergent-insoluble aggregates, only the
accumulated NF-H protein in the cytoplasm of Sf9 cells could be extracted with 0.1% Triton X-100 before fixation. Neither a filamentous structure nor protein aggregates were observed in the cytoplasm after Triton X-100 extraction (data not shown). However, examination of LH-virus-infected Sf9 cells revealed less organized parallel bundles compared with Del 1 and wild-type NF-H. The other mutants + NF-L formed short fragments, most of which were similar to those formed by NF-L alone, while a few short filaments were arranged in a straight rod-like manner and formed small parallel bundles. We observed numerous crossbridges between the parallel 10 nm filaments in the cytoplasm in cells infected with either NF-L + L alone, while a few short filaments were arranged in a straight rod-like manner and formed small parallel bundles. We observed numerous crossbridges between the parallel 10 nm filaments in the cytoplasm in cells infected with either NF-L + Del 1, NF-L + Del 2, NF-L + Del 3 or NF-L + Del 5 virus (Fig. 5A,B,C,E). Del 4 + NF-L did not exhibit crossbridge formation (Fig. 5D). The length of the crossbridges in each transfection study was determined by statistical analysis (Fig. 6), and the difference in length according to the number of amino acid residues in the C-terminal tail domains of each mutant was noted. This finding suggests that almost all of the amino acid residues in the C-terminal tail domain are involved in crossbridge formation and filament bundling. It seems that the KSP region and the last portion of the C-terminal tail domain play a crucial role in filament elongation and bundle formation. The frequency of the crossbridges formed in wild-type NF-H or in each deletion mutant (Del 1, Del 2, Del 3 and Del 5) with NF-L was almost the same. No crossbridges were observed between adjacent filaments formed by Del 4 + NF-L (Fig. 5D). This finding suggests that the last 191 amino acid residues of NF-H tail domain are more important for the formation of crossbridges between core filaments.

**DISCUSSION**

**Coexpression of NF-L and NF-H forms parallel 10 nm filaments with crossbridge structures in vivo**

Several lines of evidence indicate that NF-L by itself can form complete, 10 nm filaments in vitro in the absence of NF-M and wild-type NF-H is involved in crossbridge formation, Sf9 cells were infected with recombinant viruses expressing both NF-L and one of the deletion mutants. The filamentous structures were examined by quick-freeze, deep-etch electron microscopy. Del 1 tended to be arranged in parallel 10 nm filament bundles, while Del 2 revealed less organized parallel bundles compared with Del 1 and wild-type NF-H. The other mutants + NF-L formed short fragments, most of which were similar to those formed by NF-L alone, while a few short filaments were arranged in a straight rod-like manner and formed small parallel bundles. We observed numerous crossbridges between the parallel 10 nm filaments in the cytoplasm in cells infected with either NF-L + Del 1, Del 2, Del 3 or Del 5 virus (Fig. 5A,B,C,E). Del 4 + NF-L did not exhibit crossbridge formation (Fig. 5D). The length of the crossbridges in each transfection study was determined by statistical analysis (Fig. 6), and the difference in length according to the number of amino acid residues in the C-terminal tail domains of each mutant was noted. This finding suggests that almost all of the amino acid residues in the C-terminal tail domain are involved in crossbridge formation and filament bundling. It seems that the KSP region and the last portion of the C-terminal tail domain play a crucial role in filament elongation and bundle formation. The frequency of the crossbridges formed in wild-type NF-H or in each deletion mutant (Del 1, Del 2, Del 3 and Del 5) with NF-L was almost the same. No crossbridges were observed between adjacent filaments formed by Del 4 + NF-L (Fig. 5D). This finding suggests that the last 191 amino acid residues of NF-H tail domain are more important for the formation of crossbridges between core filaments.
and/or NF-H (Geisler and Weber, 1981; Liem and Hutchison, 1982). Following the addition of NF-M or NF-H, many thin side arms can be observed to project from the core filaments (Hisanaga and Hirokawa, 1988). The expression of NF-L alone in insect Sf9 cells can induce the formation of a loose network of 10 nm filaments in which no crossbridges are observed (Nakagawa et al., 1995). Immunocytochemical studies using specific antibodies suggest that the head and rod domains of NF-L, NF-M and NF-H form the filament core, and the long C-terminal tail domains of NF-M (439 amino acids) and NF-H (660 amino acids) project from the core filament to form crossbridges (Hirokawa et al., 1984; Balin et al., 1991; Mulligan et al., 1991). However, disruption studies of NF-H revealed that in peripheral motor and sensory axons, absence of NF-H does not significantly affect the number of neurofilaments for axonal elongation or targeting. Loss of NF-H caused only a slight reduction in the nearest-neighbor spacing of neurofilaments and did not affect neurofilament distribution in either large or small axons. It seems that while the NF-M tail domain governs the nearest-neighbor spacing of the neurofilaments, NF-H is not necessary for it. However, our experiments provide direct evidence to indicate that the C-terminal tail domain of NF-H forms the
crossbridge structures between adjacent neurofilaments in vivo, as in the case of axons (Hirokawa, 1982; Hirokawa et al., 1984). The lengths of the crossbridges between neurofilaments were nearly the same as those formed in Sf9 cells following the coexpression of NF-L and NF-M (Nakagawa et al., 1995), even though NF-H has a longer tail domain. This strongly suggests that both NF-M and NF-H participate in the determination of the nearest-neighbor distance between adjacent neurofilaments by forming crossbridges.

In vitro reconstitution studies have revealed that the average length of the projections in the filaments formed by NF-L and NF-H is about 63 nm, while that formed by NF-L and NF-M is 55 nm (Hisanaga and Hirokawa, 1988). The difference in the lengths of the projections may reflect the difference in the length of the C-terminal tail domain between NF-M and NF-H. However, our results showed that there is no difference in the lengths of the crossbridges between those formed by NF-M or NF-H coexpressed with NF-L in Sf9 cells. NF-H null mice also did not exhibit any alteration in the density of neurofilaments in motor and sensory axons. However, in this regard, one noteworthy finding is the difference in phosphorylation level of the tail domain of NF-M and NF-H in the different expression systems. It is thought that phosphorylation of the tails of NF-M and NF-H increases the total amount of negative charge, and thus the degree of lateral extension of their C-terminal side arms (Glicksman et al., 1987; Myers et al., 1987), which in turn causes increased NF neurofilament spacing.

Unmyelinated initial axonal segments that contain dephosphorylated NF-H have a higher filament density than myelinated ones (Nixon et al., 1994). A trembler mouse with a decreased phosphorylation level of NF-H was shown to have an increased NF density (de Waegh et al., 1992). NF-M expressed in Sf9 cells was recognized by specific antibody that recognizes its phosphorylated form. Its molecular weight, as determined by SDS-PAGE, was as high as that of normal NF-M in axons. This means that it has a strong ability to assemble with NF-L in Sf9 cells. Although the lengths of the crossbridges formed by the tail domain of NF-M were shorter than those of the side arms in reconstitution experiments in vitro, this could be due to some motifs being used for binding other core filaments. In the case of coexpression of NF-L and NF-H in Sf9 cells, the molecular weight of NF-H was approximately 180 kDa, which is higher than its true molecular weight (115 kDa), but lower than that of its phosphorylated form (200 kDa) in axons. These suggest that while some phosphorylation sites are phosphorylated, others are not. Neurofilament function depends on the state of phosphorylation of the numerous serine/threonine residues in NF-M and NF-H. Most phosphorylation occurs in the Lys-Ser-Pro (KSP) repeats in the C-terminal tail domains. CDK5 has been shown to phosphorylate KSPXK repeats in neurofilament protein (Lew et al., 1992; Hisanaga et al., 1993; Veeranna et al., 1995, 1996; Guidato et al., 1996; Sun et al., 1996), and mitogen-activated protein kinases (Erk1, Erk2) have been shown to phosphorylate all types of KSP motifs in peptides (KSPXK, KSPXXK, KSPXXXXK and KSPXXXXK) derived from NF-M and NF-H. In our experiments, although some KSP motifs may be phosphorylated by unknown kinases in Sf9 cells, others may not. Thus, it is plausible that in Sf9 cells infected with the LH virus, the C-terminal region of NF-H is partially phosphorylated. The results showing that the lengths of the crossbridges in LM-virus-infected cells and LH-virus-infected cells are almost the same may be due to either a partially phosphorylated C-terminal region of NF-H not being fully extended, or to longer motifs at the very terminal of the C-terminal part of NF-H being used for binding adjacent core filaments.

**Fig. 4.** Quick-freeze, deep-etch electron microscopy image of the filaments formed by NF-L and NF-H in transfected Sf9 cells. These correspond to the ultrathin-section view in Fig. 3B (LH virus). Frequent crossbridges (arrows) were observed between the 10 nm filaments arranged in parallel.
NF-H domains that regulate crossbridge formation and its length, and longitudinal elongation of the core filaments

The expression of NF-L in Sf9 cells induces the formation of only short fragmented filaments that are arranged in a random 10 nm filament network. Coexpression of NF-L and NF-H induces the formation of bundles of 10 nm filaments packed in a parallel manner with frequent crossbridges resembling the neurofilamentous domain in the axon, although the bundles were not as long as those formed by NF-L and NF-M. Our deletion mutant studies of the NF-H tail domain reveal that this domain is an actual component of crossbridge structures, as in the case of NF-M. Del 1 did not exhibit impaired bundle-forming ability. Del 2, which lacks the N-terminal half of the KSP repeat motifs, showed a poorer ability to organize parallel filamentous bundles than Del 1 and wild-type NF-H, while parallel filament bundles were much more frequently observed in Del 2 compared with other mutants. Other mutants + NF-L formed short fragmented filaments, most of which were similar.

Fig. 5. Quick-freeze, deep-etch electron microscopy images of the filaments formed by NF-L and each of the deletion mutants of NF-H. Frequent crossbridges (arrows) were observed between adjacent 10-nm filaments in Sf9 cells cotransfected with NF-L + Del 1 (A), NF-L + Del 2 (B), NF-L + Del 3 (C) and NF-L + Del 5 (E). In NF-L + Del 4 (D)-transfected Sf9 cells, short randomly organized filaments were observed. Scale bar, 200 nm.

Fig. 6. The lengths of crossbridges formed by wild-type NF-H and each of its deletion mutants were measured. Del 4 did not form crossbridges. Del 1, lacking 97 amino acids between the rod domain and KSP repeats in the C-terminal tail domain, formed crossbridges that were slightly shorter than those formed by wild-type NF-H. Del 2-3, lacking part or all of the KSP repeats, and Del 5, lacking the entire KSP region and 97 amino acids between the rod domain and KSP region, formed apparently short crossbridges. These data suggest that the tail domain of NF-H is the actual component of the protein in the crossbridge structures.
to those formed by NF-L alone, but a few short filaments were arranged in a straight rod-like manner and formed small parallel bundles.

For all intermediate filament subunits, variable head and tail domains flank the conserved alpha-helical rod domain. The rod domain is subdivided by a short nonhelical linker to form coil 1 and coil 2. Except for NF-M, NF-H and lamina that are localized below the inner nuclear membrane and form a nuclear lamina in most eukaryotic cells, coil 1 is further subdivided into coil 1a and coil 1b by another nonhelical linker (Lee and Cleveland, 1996). This implies that coil 1 of the rod domain of NF-M and NF-H is a continuous coil that may affect the flexibility of the filaments. This could explain why some of the filaments were not so flexible when assembled with NF-L, although the filaments were very short compared with those assembled with wild-type NF-H and NF-L. It seems that the rod domain of NF-H influences the flexibility of the neurofilaments and the C-terminal tail domain forms crossbridges and helps the filament to elongate longitudinally, as in the case of NF-M (Nakagawa et al., 1995).

Quick-freeze, deep-etch electron microscopy revealed frequent crossbridges in deletion mutants Del 1 + NF-L, Del 2 + NF-L, Del 3 + NF-L and Del 5 + NF-L, similar to those formed by wild-type NF-H + NF-L. In contrast, the filaments formed by Del 4 + NF-L show loose arrangement with few crossbridges. This suggests that the last 191 amino acid residues of the C-terminal tail domain are a prerequisite for crossbridge formation. Deletion mutants Del 1, Del 2 and Del 3 lacked the first 97 amino acids or KSP repeats, resulting in different lengths of the crossbridges; thus, the last 191 amino acid residues may affect both the length and the binding of the tail domain to another core filament or some motifs on the tail domain of adjacent filaments. These results suggest that the main binding region in the tail domain of NF-H to another neurofilament or microtubule is located in the last 191 amino acid residues.

In our experiments, transfection of the deletion mutants with NF-L resulted in the formation of only a few short filaments in transfected non-neuronal cells. The C-terminal tail domain is subdivided by a short nonhelical linker to form coil 1 and coil 2. Except for NF-M, NF-H and lamina that are localized below the inner nuclear membrane and form a nuclear lamina in most eukaryotic cells, coil 1 is further subdivided into coil 1a and coil 1b by another nonhelical linker (Lee and Cleveland, 1996). This implies that coil 1 of the rod domain of NF-M and NF-H is a continuous coil that may affect the flexibility of the filaments. This could explain why some of the filaments were not so flexible when assembled with NF-L, although the filaments were very short compared with those assembled with wild-type NF-H and NF-L. It seems that the rod domain of NF-H influences the flexibility of the neurofilaments and the C-terminal tail domain forms crossbridges and helps the filament to elongate longitudinally, as in the case of NF-M (Nakagawa et al., 1995).

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In our experiments, transfection of the deletion mutants with NF-L resulted in the formation of only a few short filaments in a parallel array, except transfection of Del 1 and Del 2. That is, deletion mutants lacking the latter half or all of the KSP repeat sequence motifs or the last 191 amino acid residues may lose the ability to form long thick filament bundles. Most of them are coordinated in a random manner, forming loose networks. It is evident that the binding domain is a prerequisite for crossbridge formation, but the KSP repeats and other sequence motifs before the binding domain also contribute to the enhancement of the interaction force between the filaments. Thus, the results of our experiments suggest that the tail domain from the KSP repeat motifs is involved in crossbridge formation and longitudinal elongation. Whether the binding domain on the tail domain directly interacts with another core filament or with the tail domain of an adjacent filament to form crossbridges remains unclear.

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