Neurofilament (NF) Assembly; Divergent Characteristics of Human and Rodent NF-L Subunits*

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Intermediate filaments (IFs)1 are a heterogeneous family of proteins sharing common structural features that can be subdivided into six types (I–VI) based on sequence homology (1). IF genes are expressed in a cell type-specific and developmentally regulated manner with cells frequently containing only a single IF type at a particular stage of differentiation. Neurofilaments (NFs) are the predominant IF in mature neurons but are preceded during neuronal differentiation by a succession of other IFs including vimentin (2) nestin (49), α-internexin (3, 4), and peripherin (5). Neurofilaments are assemblies of three subunits, the NF-L (molecular mass, 68 kDa), NF-M (150 kDa), and NF-H (200 kDa) (6). These three components form heteropolymeric 10-nm filaments that run parallel along the length of the axon with frequent cross-bridges between neighboring filaments. Axonal neurofilaments are thought to serve a primarily structural function. Evidence from a Japanese quail (quisquer) with a spontaneous mutation in NF-L (7) and a line of transgenic mice expressing an NF-H-β galactosidase fusion protein (8) suggest that a loss of axonal neurofilaments results in a decreased axonal diameter.

The first step in filament formation is the lateral associations of the α-helical rod domains via hydrophobic interactions to form a coiled-coil dimer (9). The rod consists of an α-helix that is interrupted by three short non-helical linker sequences (L1, L12, and L2). Heptad repeats of hydrophobic amino acids confer an amphipathic character to the α-helical domain that allows coiled-coil interactions between compatible IF molecules. This may result in homodimer formation in the case of vimentin or obligate heterodimer formation in the case of type I and type II keratins (9).

Both in vitro and in vivo studies have probed the ability of individual neurofilament proteins to form homo- and heteropolymers. Purified bovine (10–16), porcine (17, 18), and murine NF-L (19) and, to a lesser degree, NF-M and NF-H (10, 14) assemble in vitro into 10-nm homopolymers (20). In contrast, rodent neurofilament proteins expressed individually in cells that lack an endogenous intermediate filament network (SW13 vim− cells) are unable to form homopolymers, yet can form 10-nm filaments when coexpressed with NF-M or NF-H (21, 22) or when expressed in cells containing an endogenous vimentin network, through assembly with vimentin (21, 23, 24).

In the present work we examine the human NF-L and NF-M and derivatives of them in SW13 (vim-) cells and in the yeast “interaction trap” system. Our results demonstrate that human NF-L is capable of self-assembly, an important distinction from the rodent NF-L subunits which are obligate heteropolymers. Human NF-M or truncations of it that contained the rod domain could not polymerize in the absence of NF-L. Our analysis shows that two distinct structural features of the human NF-L rod are important determinants of its ability to homodimerize. First, human NF-L has more charged residues next to the hydrophobic residues of the heptad repeats in its rod domain.

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1 The abbreviations used are: IFs, intermediate filaments; aa, amino acids(s); NF, neurofilament(s); PBS, phosphate-buffered saline.
proline residue allows the L12 linker region of human NF-L to mimic an α-helix with a heptad repeat. The same region of rat NF-L contains the additional serine, and its conformation is predicted to be an extended β-structure (25). Since dimer formation is likely to be the initiating event in filament formation, our results suggest that this important intracellular event may be different in human than in rodents.

EXPERIMENTAL PROCEDURES

Neurofilament Clones and Their Derivatives—The plasmid pNF-L contains a complete human NF-L gene plus 2.8 kilobase pairs of upstream sequences. The plasmid pRSVNF-L containing a full-length rat NF-L cDNA driven by a Rous sarcoma virus promoter was obtained from Dr. L. R. Liem (21). A plasmid containing a complete human NF-M genomic clone (pNF-M) has been previously described (26). To create a “tagged” human NF-M, a nucleotide sequence encoding an 11 amino acid epitope tag (Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg) was inserted at either amino acid 21 (amino-terminal tagged, designated pS8919) or amino acid 444 (internal tagged, designated pS8928) of the plasmid pNF-M (numbering according to Ref. 50).

The internal tagged NF-M plasmids were used to prepare the following series of deletions: amino-terminal domain (amino acids 22–80 deleted); glutamic acid-rich domain (aa 451–611 deleted); glutamic acid–multiphosphorylation repeat (aa 551–790 deleted); and a carboxyl-terminal domain (aa 690–914 deleted). An NF-M/L-M hybrid gene was constructed by removing amino acids 89–444 from pNF-M and replacing it with a polymerase chain reaction-amplified NF-L rod domain (aa 83–414). The rod region of the final product was verified by sequencing.

For yeast interaction trap experiments, fragments encoding NF rod domains (human NF-L amino acids 85–410, human NF-M amino acids 98–421, and rat NF-L amino acids 86–415) or full-length proteins were cloned into the vectors pGBT9 and pGAD42 (27) using oligonucleotides to preserve open reading frames.

Site-specific Mutagenesis—Amino acids were inserted, deleted, or substituted using the Quickchange site-directed mutagenesis kit (Stratagene). Changes were verified by sequencing on an Applied Biosystems automated DNA sequencer.

Tissue Culture and DNA Transfection—Human SW13 (vim+ or vim−) cells were obtained from Dr. R. Evans and cultured as described (28). Cells were transiently transfected by electroporation (440 V, 500 microfarads) and cultured as described (28). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and streptomycin. The final product was verified by sequencing.

RESULTS

The Human NF-L Subunit Is Capable of Homopolymerization in Vivo—The ability of human NF-L to self-assemble into filaments was assessed in human SW13 (vim−) cells that lack an endogenous intermediate filament network (28). These cells were transfected with a plasmid (pNF-L) containing the entire human NF-L gene plus 2.8 kilobase pairs of upstream sequence. Under our conditions we obtained an average transfection efficiency of 15–20%. NF-L and vimentin expression was monitored by immunofluorescence 48 h following transfection.

Transfection of human NF-L alone resulted in extensive filament formation throughout the cytoplasm in the absence of any other IF protein (Fig. 1A). Staining with anti-vimentin antibodies revealed that 1–4% of the cells were vimentin positive. Others (22) have reported comparable levels of spontaneous reversion in this cell line. However, double labeling with antibodies to NF-L and vimentin demonstrated that filament formation by human NF-L was not dependent on the presence of vimentin (data not shown). These results contrast sharply with those reported previously for rodent NF subunits which showed that the rat and mouse NF-L proteins do not assemble into filaments under similar conditions (21, 22). This difference between cell types and those of others can be attributed to systemic or technical differences in the experiments. We have transfected SW13 (vim−) cells with the rat nf-l gene used by Ching and Liem (21) and obtained results that are in complete agreement with theirs: the rat NF-L protein distributed uniformly throughout the cytoplasm and no filamentous structures present (Fig. 1B). Thus, differences in the ability of hu-

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Note that the turn before the Pro-kink assumes a conformation close to a Δ3 helix.
man and rodent NF-L to homopolymerize appear to reflect potentially important and fundamental differences that are traceable to the few differences in the amino acid sequences of the two proteins.

**Human NF-M Is Unable to Homopolymerize in Vivo**—We carried out similar transfection experiments using human NF-M to determine if the ability to homopolymerize was present in other human NF subunits or was restricted to NF-L. The plasmid pSS028 contains a modified human NF-M gene that encodes an NF-M protein containing an 11-amino acid tag sequence (Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg) inserted at amino acid 444 (internal tag). The inclusion of this epitope tag allowed the simultaneous detection of both phosphorylated and non-phosphorylated forms of the protein with an anti-tag monoclonal antibody. Our previous studies have demonstrated that the tag does not interfere with expression or polymerization of NF-M in transgenic mice or transfected cells and that filaments containing the tag appear to function normally (37).

Expression of the tagged human NF-M protein in SW13 (vim−) cells did not result in the formation of filamentous networks. Immunofluorescence staining using anti-tag antibody revealed that the human NF-M protein was uniformly distributed throughout the cytoplasm in a diffuse and at times granular pattern (Fig. 1C). In this regard, the human NF-M behaves similarly to the rodent NF-L and NF-M subunits.

**Human NF-M and Rat NF-L Form Filaments in Vivo When Expressed Together or with Human NF-L**—Interestingly, rat NF-L and human NF-M can participate in filament formation through heteropolymerization. Double transfection experiments were performed in which human NF-M was coexpressed with either human or rat NF-L. Cells transfected with human NF-L and human NF-M contained an extensive filamentous network throughout the cytoplasm that contained both NF subunits co-localized within filaments (Fig. 2, A and C). In Fig. 2C, the finer filaments corresponding to those labeled with antibodies directed at the human NF-L do not appear in the photomicrograph because the gain of the photomultiplier was set so that the main fibers reveal good detail. At higher gain settings the full array of fine filaments was visible. Expression of rat NF-L with human NF-M also resulted in extensive filament formation and co-localization of both proteins (Fig. 2, B and D) indicating the assembly competence of each subunit, but only as a heteropolymer.

**Deleting the Amino- or Carboxyl-terminal Sequences Flanking the Human NF-M Rod Domain Does Not Result in NF-M Homopolymerization**—Inhibitory regions in parts of the molecule not directly involved in homophilic interactions could explain the incompetence of human NF-M to homopolymerize. To address this possibility, NF-M genes encoding truncated forms of the tagged human protein were prepared and transfected into SW13 (vim−) cells. The following series of deletions were examined: Δamino-terminal domain (amino acids 22–80 deleted); Δglutamic acid-rich domain (aa 451–611); Δglutamic acid-rich and multiphosphorylation repeat (aa 551–790); Δcarboxy-terminal domain (aa 690–914). Expression of these truncated proteins was assessed using anti-tag monoclonal antibodies and, where appropriate, the anti-NF antibodies SMI-31 and SMI-32 which detect phosphorylation-dependent and independent epitopes of the human NF-M, respectively (38). In all cases staining was diffuse and distributed throughout the cytoplasm without evidence of filament formation (data not shown). However, none of the deletions affected the ability of human NF-M to form heteropolymers since filaments formed when the deletions were coexpressed with human NF-L (data not shown).
Interaction-trap analysis of various neurofilament subunits and their rod domains

The values shown are the relative β-galactosidase activities expressed in doubly transfected yeast cultures after normalization for cell number. Plasmids containing the transactivating domain begin with T (e.g., T-hM), and plasmids containing the DNA binding domain begin with D (e.g., D-hM). Abbreviations: hM, human NF-M; hM rod, human NF-M rod; hL, human NF-L; hL rod, human NF-L rod; rL, rat NF-L; rL rod, rat NF-L rod.

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| Fig. 3. Alignment of human and rat NF-L rod domains. Positions of α-helical regions 1A, 1B, 2A, and 2B, and linker regions L1, L2, and L12 are indicated. Sequence differences between rat and human (⁎) are indicated. The bold symbols represent amino acids that are referred to in the text. |

Table II

The failure of human NF-M to form homopolymeric filaments might indicate the incompetence of its rod domain to form dimers. If so, then replacement of the NF-M rod with the human NF-L rod should allow homopolymerization of the hybrid molecule. To test this hypothesis a hybrid NF-M-L-M gene was created which encodes a protein with the amino- and carboxyl-terminal domains of NF-M but the rod domain of human NF-L. When this construct was expressed alone in SW13 (vim) cells, a diffuse granular staining was apparent throughout the cytoplasm, and no filament assembly was detected. However, when the NF-M-L-M protein was coexpressed with human NF-L, extensive filament formation occurred. These results indicate that the amino- and carboxyl-terminal sequences of NF-M might interfere with homopolymerization, but the failure of the native NF-M to homopolymerize cannot be solely attributed to this interference since the NF-M rod domain without flanking sequences does not exhibit significant homophilic interactions in the yeast interaction trap analyses.

The α-Helical Rod Domains of Human NF-M and Rat NF-L Are Incapable of Homodimerization—The immunocytochemical analyses described above show that human NF-L, but not NF-M, is capable of homopolymeric assembly into filaments of a dimension and complexity that can be visualized by light microscopy. Filament assembly is a multistep process, and the block to NF-M homofilament assembly might occur at steps after the initial interactions between neurofilament subunits. We investigated the earliest steps in NF-subunit interactions using the yeast "two-hybrid" or interaction trap system, similar to that used by Meng et al. (39) to investigate homodimer formation by vimentin. In this system, expression of a reporter gene (Escherichia coli lacZ gene) is controlled by a transactivator complex consisting of two hybrid proteins. A functional transactivating complex is achieved when a DNA binding domain, supplied by one hybrid subunit, and an activation domain, supplied by the second, are held in close proximity by the adhesive domains of two hybrid proteins. We examined the interactions between various NF subunits of the rod domains by incorporating them as the adhesive components in the two-hybrid complex. cDNA sequences encoding human NF-M, human NF-L, rat NF-L, or only the α-helical rod domains of each were separately fused to the activation or DNA binding subunits of the transactivating complex. All pairwise combinations of activation subunits with DNA binding subunits were tested for interactions by double transfections into yeast. The results of these experiments are summarized in Table II, where strongly positive results are presented in bold type. The results shown in the first two vertical columns indicate that full-length human NF-M or its rod domain interact strongly with only full-length human NF-L or its rod domain. All combinations of human NF-M (rod domain or whole) with itself or rat NF-L (rod domain or whole) failed to interact. These results suggest that the yeast two-hybrid assay is a more stringent test of interaction than the SW13 transfection assay, since the human NF-M was able to form filaments with the rat NF-L in the latter assay (Fig. 2, B and D). It is possible that the yeast two-hybrid system places steric constraints on the association that are not necessary for filament formation. The third and fourth vertical columns show that full-length human NF-L or its rod interact very strongly with the whole subunits or the rod domains of human NF-L, human NF-M, or rat NF-L. The last two columns indicate that rat NF-L or its rod domain interact with human NF-L or its rod domain while failing to interact with itself or human NF-M or its rod domain. The results shown in Table II were obtained with permeabilized yeast cells and have been normalized for differences in culture turbidity. It should be noted that each NF domain was tested as a fusion partner in both the activation and DNA binding subunits, and all pairwise combinations were tested for transactivation. The results of these tests do not show any significant bias between reciprocal pairs of hybrid proteins. We conclude from these analyses that human NF-L but not rodent NF-L or human NF-M is capable of significant homodimer formation.

The Charge Interaction Provided by Arg161 May Stabilize the Coiled-coil Associations of Human NF-L—Based on theoretical and biophysical considerations, we attempted to determine which of the amino acid differences between human and rat NF-L could be responsible for the differences in their homopolymerization ability. Fig. 3 shows alignments of the human and rat NF-L rod domains and indicates the position of the α-helical (1A, 1B, 2A, and 2B) and linker (L1, L2, and L12) regions (25). The human and rat NF-L rod sequences are highly homologous with only 8 amino acid differences (indicated by a * in Fig. 3). Two regions in particular were noted which could account for the difference in homopolymerization. The first was at aa 161 in the human protein which is an arginine and the corresponding aa position 162 in the rat which is a glutamine. This difference occurs at position e of the heptad repeat, where it is likely to affect the corresponding electrostatic attractions between two rod domains forming a coiled-coil. Such charged residues (usually Arg, Lys, or Glu) are considered to be responsible for additional attractive interactions between helices forming a coiled-coil (34) and are also major contributors to the characteristic stagger for a given protein.

To test this hypothesis an arginine was substituted for the glutamine at position 162 in the rat rod. The reciprocal substitution of Arg161 for Gln161 was carried out in the human NF-L rod. When transfected into SW13 (vim) cells, the human NF-L (Gln161) did not form filaments (Fig. 4A), whereas the rat NF-L (Arg162) formed filaments (Fig. 4B). We investigated whether these changes in polymerization could be traced to differences...
in the abilities of the rod domains to form dimers. The mutated rod domains of both human and rat NF-L were expressed in the yeast two-hybrid system and tested for interactiveness (Table III). Productive interactions occurred with the mutated rat NF-L (Arg162) rod domain but not with the human NF-L (Gln161) rod domain. These results show that a single amino acid change in the rod domain of either protein is sufficient to convert rat NF-L into a subunit capable of homopolymerization and human NF-L into a protein that is no longer able to self-assemble.

The L12 Linker Regions of the NF-L Subunits Affect Homopolymerization—The second difference identified as potentially important in the altered dimerization properties of the two proteins was in the L12 linker region. A comparison of rat and human NF-L sequences in this region reveals a major difference between them produced by a serine insertion in the rat sequence. To assess the importance of this difference for filament formation, we prepared reciprocal mutations in the two NF-L rod domains as follows: Ser252 was deleted from rat NF-L, making it more human like, and a serine residue was inserted into human NF-L (following Thr251) to make it more rat like. Each mutant was transfected into SW13 (vim+) cells. Human NF-L with the inserted serine failed to homopolymerize (Fig. 4C), whereas rat NF-L with the serine deleted formed filaments (Fig. 4D). Analysis of these mutations in the yeast two-hybrid system supported the conclusions of the transfection experiment and demonstrated that rat NF-L with Ser252 deleted dimerizes, whereas human NF-L with the added serine did not (Table III).

The L12 linker region is generally believed to assume an extended β-sheet conformation (25). However, structural and biophysical considerations led us to investigate whether the L12 region of the human NF-L rod domain might behave like a coiled-coil due to the heptad repeat-like sequence in this region (Fig. 5). One striking characteristic of human NF-L and several other IFs capable of homopolymerization is the absence of a serine at position 252 that is present in rat NF-L. Human NF-L and other IFs also have a proline residue that is highly conserved at position b in the following heptad (Fig. 5). In α-helices, prolines are well known to disrupt the helical hydrogen bonding pattern and form a “Pro-kink” (35, 40, 41). A characteristic structural distortion produced by the Pro-kink is a “face shift” that causes the helical portions before and after the proline to rotate with respect to each other, thereby interfering with local helical periodicity (32, 35, 41). Since the orientation of the hydrophobic residues in a heptad is a key element in coiled-coil formation, the face shift due to the Pro-kink may interfere with the appropriate helix-helix interface (i.e. the dimerization surface). However, structural considerations indicate that the deletion of a residue from the heptad preceding the proline in the human sequence (compared with the rat sequence) would compensate for the face shift by bringing the two sets of hydrophobic residues back to one face that incorporates the Pro-kink. This is illustrated in Fig. 6 where purple and orange spheres indicate the dimerization surfaces in the mutated Pro-kinked helix compared with an ideal α-helix. We hypothesized that the serine deletion is therefore necessary for the human NF-L helices to dimerize, and we tested this hypothesis by assessing the homopolymerization abilities of three different rat NF-L mutants. In the first construct described above, we deleted Ser252 in rat heptad 3 (Fig. 5) that precedes Pro254 to bring the hydrophobic residues to the same face of the two helices (Fig. 4D; Table III). However, in itself a dimerization of this construct would not necessarily prove the existence of a coiled-coil in the region since other structural explanations are feasible. We probed the existence of a coiled-coil in the region by making two more mutant NF-L proteins. The Pro254 of rat NF-L was mutated to an Ala254 to remove the kink and bring positions a and d onto the same face of the helix. However, the heptad (heptad 3) preceding Pro254 contains an aspartic acid at position d in the rat replacing a hydrophobic residue commonly at this position of a canonical heptad (34). Consequently, we also changed Asp249 to Leu249 to produce a

![Fig. 4. Immunofluorescent assessment of the homopolymerization of mutant rat and human rod domains. SW13 cells were transfected with relevant NF-L genes and immunofluorescent stained using rabbit polyclonal anti-NF-L primary antibody followed by donkey anti-rabbit fluorescein-conjugated secondary antibody. Immunofluorescent staining was analyzed by laser scanning confocal microscopy. A, human NF-L Gln161; B, rat NF-L Arg162; C, human NF-L + Ser252; D, rat NF-L ΔSer252. Scale bar, 10 μm.](Image 56x486 to 300x729)

![Table III. Homodimer interaction trap analyses of mutant rat and human NF-L rod domains.](Image 313x681 to 561x729)

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Values shown are the relative β-galactosidase activities in yeast cultures doubly transfected with plasmids encoding GAL4 DNA binding and GAL4 activation domains, each fused to the same rat or human NF-L rod domain. rL and huL are wild type domains; R-Q, Q-R substitution of R for Q and Q for R; ΔS, +S deletion and insertion serine.
Divergent Characters of Human and Rodent NF-L Subunits

In mature neurons, the three neurofilament proteins copolymerize to form 10-nm filaments. These filaments form bundles consisting of “core filaments” interconnected by cross-bridges not found in filaments composed of other IF proteins. When neurofilaments are assembled in vitro all three NF subunits are incorporated integrally into the filament cores, whereas the carboxyl-terminal tail sequences of NF-M and NF-H extend away from the filament surface and are the major constituents of cross-bridges (11, 42). In vitro studies have long suggested that homopolymerization can be achieved with NF-L alone while under most conditions NF-M and NF-H do not assemble into homopolymers (10–15, 17, 18).

However, the relevance of in vitro to in vivo filament assembly has recently been challenged. Two groups using the SW13 (vim<sup>−</sup>) cell line have shown that unlike type III IFs (such as vimentin), none of the rat or mouse NF subunits can form filaments when transfected individually into cells lacking an endogenous IF network (21, 22). Nevertheless, all three NF subunits can co-assemble with α-internexin (21), and NF-L can combine with either NF-M or NF-H to form filamentous networks. Transfection experiments in the insect cell line Sf9 which also lacks endogenous IFs have yielded similar results (42); when rat NF-L was expressed alone no filamentous staining was observed but when expressed together with NF-M it co-assembled to form bundles of 10-nm filaments with frequent cross-bridges resembling axonal neurofilaments (21). These results convincingly demonstrate that rodent neurofilaments are obligate heteropolymers in vivo requiring NF-L plus either NF-M or NF-H. The present study used a similar design to investigate the assembly properties of the human NF-L and NF-M subunits and demonstrates the distinctive ability of human NF-L to homopolymerize, a finding that distinguishes human NF-L from human NF-M and its rodent NF-L counterpart.

To investigate the level at which the block to filament formation occurs we utilized the yeast interaction trap system. Since the yeast interaction trap requires direct protein–protein interactions for activation of a reporter gene, it should measure the first step in filament production, i.e., dimer formation (39). The inability of human NF-M or rat NF-L to self-interact productively in this assay argues that homopolymerization of these subunits is blocked at the first step of assembly. Conversely the positive interaction between human NF-L fusion pairs is consistent with this subunit’s ability to form homopolymers in vivo in transfected cells. Our results suggest that neurofilaments found in the Triton X-100-insoluble fractions of SW13 (vim<sup>−</sup>) cells transfected with rat NF-L (21) may not be true homopolymers of NF-L but rather associations that are nonspecific or of insufficient strength to be scored by the yeast system. However, it is not clear how to reconcile our results with reports that a truncated rat nf-l gene containing amino acids 24–542 can productively self-interact in the yeast two-hybrid system (43). Conceivably the particular truncation is important in conferring this ability.

Two obstacles likely exist to the homopolymerization of human NF-M. First, the failure of human NF-M to self-interact in the yeast two-hybrid system suggests that the NF-M rod domain itself is incompetent to homodimerize. However, replacement of the NF-M rod domain with one known to be competent to dimerize (human NF-L rod) did not produce a protein (NF-M-L-M) that homopolymerizes. Yet when the NF-M-L-M hybrid was coexpressed with normal human NF-L, there was extensive filament formation. These findings indicate a strong influence of flanking sequences on subunit association. Thus, homopolymerization of NF-M is prevented by two factors, each of which is strong enough by itself to prevent homoassociation, but nonetheless the protein retains the ability to heteropolymerize. These observations suggest a previously unsuspected role of the flanking sequences in neurofila-
ment assembly, preventing certain associations while allowing others.

Additionally these studies make two observations relevant to the general process of intermediate filament assembly. First they show that a single amino acid change in one heptad repeat can drastically alter the dimerization potential of a rod domain. The sequence similarities in the rod domains of homodimerizing and non-homodimerizing NF subunits is very high. In NF-L there are only 8 amino acid differences in the 310 residues of the human and rat rod domains, and we show that a single change in the rat rod domain (Gln162 to Arg162) is sufficient to allow robust homopolymerization. This substitution appears at position \( e \) which is generally occupied by charged residues (usually Arg, Lys, or Glu) in a regular heptad characteristic of coiled-coils (25). The residues in this position are responsible for additional attractive interactions between the helices forming the coiled-coil (34). Thus replacing the rat Gln with an Arg renders this heptad more “ideal” and was sufficient to allow robust homopolymerization. The failure of the human NF-L to homodimerize following the reciprocal substitution at Arg161 in human with the Gln present in rat demonstrates the importance of this minor change on the overall ability of the \( \alpha \)-helical rods to dimerize.

Homodimerization appears to have more stringent requirements than heterodimerization and may demand the most perfect set of heptad repeats. If the ability of an IF rod domain to homodimerize reflects the summed homodimerizing capacities of each individual heptad, then human NF-L may be just above a threshold of compatibility needed for homodimerization while the rat NF-L may be just below it. Thus making even a single heptad more ideal in the rat converts it into a homodimerizing subunit while making one heptad less perfect converts the human into a subunit incapable of homodimerizing.

A second finding likely relevant to the assembly of many homopolymeric IFs is the critical role of the L12 linker region. As illustrated in Table IV, intermediate filament subunits that homopolymerize have a characteristic L12 sequence that includes a highly conserved proline and a spacing of 25 residues between the last full heptad repeat of the 1B segment and the proline residue in the L12 region. Those subunits known to be obligate heteropolymers have acquired an additional amino acid residue or have undergone significant sequence diver-
gence. The net effect of the observed insertion relative to the human sequence is to rotate, with respect to one another, the hydrophobic patches of the helix before and after the conserved proline, such that they are no longer on the same face of the helix (Fig. 6). The change in dimerization surface resulting from this rotation weakens the interactions between subunits due to the inability to form a sufficiently stable coiled-coil association. We have demonstrated that rotating the positions of the helix before and after the conserved proline of rat NF-L relative to one another, either by a serine deletion or by the creation of a canonical α-helical heptad repeat by replacing the proline and preceding aspartic acid, results in a protein that can homopolymerize. Thus, the L12 region of the human NF-L exhibits the structural interaction properties expected of an α-helix with a heptad repeat and aligns the heptad repeat before and after it, allowing homopolymerization of the subunit.

We do not know the biological significance, if any, of the human NF-L’s ability to homodimerize. The human subunit has evolved or preserved the same primary sequence characteristics of other homopolymerizing intermediate filaments. The ability of subunits to homo- or heteropolymerize may shape the course and character of filament formation and turnover. If rodent NF-L subunits can only heterodimerize and human NF-L can hetero- and homodimerize, the composition of neurofilaments may differ between species. Such an assembly model predicts that the mole fraction of NF-L in rodent neurofilaments must be $\frac{1}{2}$ (assuming that NF-M and NF-H do not form dimers with each other or with themselves). Conversely human NF-L, which is unconstrained in its choice of partners and can form homo- as well as heterodimers, may have a mole fractions between $\frac{1}{2}$ and 1. Unfortunately the molar compositions of filaments reported in the literature varies widely (44–47) and cannot be used to verify these predictions. Moreover, since distinct subpopulations of neurons may possess filaments with different subunit compositions, species differences may only be apparent when cognate subpopulations are compared.

Yet whatever functional significance these findings may have for neurofilament assembly in *vivo*, our results clearly indicate that subtle alterations in the rod domain of an intermediate filament protein can drastically alter its assembly properties. Our results also point to the importance of the L12 linker region in filament assembly.

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