The Rod Domain of NF-L Determines Neurofilament Architecture, Whereas the End Domains Specify Filament Assembly and Network Formation

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Abstract. Neurofilaments, assembled from NF-L, NF-M, and NF-H subunits, are the most abundant structural elements in myelinated axons. Although all three subunits contain a central, α-helical rod domain thought to mediate filament assembly, only NF-L self-assembles into 10-nm filaments in vitro. To explore the roles of the central rod, the NH₂-terminal head and the COOH-terminal tail domain in filament assembly, full-length, headless, tailless, and rod only fragments of mouse NF-L were expressed in bacteria, purified, and their structure and assembly properties examined by conventional and scanning transmission electron microscopy (TEM and STEM). These experiments revealed that in vitro assembly of NF-L into bona fide 10-nm filaments requires both end domains: whereas the NH₂-terminal head domain promotes lateral association of protofilaments into protofibrils and ultimately 10-nm filaments, the COOH-terminal tail domain controls lateral assembly of protofilaments so that it terminates at the 10-nm filament level. Hence, the two end domains of NF-L have antagonistic effects on the lateral association of protofilaments into higher-order structures, with the effect of the COOH-terminal tail domain being dominant over that of the NH₂-terminal head domain. Consideration of the 21-nm axial beading commonly observed with 10-nm filaments, the approximate 21-nm axial periodicity measured on paracrystals, and recent cross-linking data combine to support a molecular model for intermediate filament architecture in which the 44–46-nm long dimer rods overlap by 1-3-nm head-to-tail, whereas laterally they align antiparallel both unstaggered and approximately half-staggered.

Neurofilaments (NFs), composed principally of the three polypeptides NF-L (apparent M, 68 kD), NF-M (apparent M, 160 kD), and NF-H (apparent M, 200 kD), are a major component of the neuronal cytoskeleton (for a review see Liem, 1993). The most abundant subunit of native neurofilaments is NF-L, which has been suggested to form a “core” filament with which NF-M and NF-H coassemble (Delacourte et al., 1980; Geisler and Weber, 1981; Liem and Hutchison, 1982; Aebi et al., 1988; Troncoso et al., 1989; Hisanaga and Hirokawa, 1988, 1989). The COOH-terminal tails of NF-M and NF-H project as “side-arms” that extend radially 55 and 63 nm, respectively, from the filament shaft (Hisanaga and Hirokawa, 1988; Troncoso et al., 1990; Gotow et al., 1992). Typical for IF structures in the electron microscope, filaments reconstituted from NF-L appear as relatively featureless 10-nm filaments after negative staining, although sometimes protofilament-like structures can also be observed (e.g., Geisler and Weber, 1981; Liem and Hutchison, 1982; Aebi et al., 1988; Troncoso et al., 1990). When glycerol-sprayed onto freshly cleaved mica and rotary metal-shadowed, the same filaments exhibit a characteristic ~21-nm axial repeat of beaded segments (e.g., Milam and Erickson, 1982; Aebi et al., 1988; Troncoso et al., 1990). The common to all IF polypeptides is a conserved “rod” domain (e.g., Geisler and Weber, 1982; Hanukoglu and Fuchs,
linking has yielded three dominant modes of antiparallel assembly for keratin (Steinert and Parry, 1993; Steinert et al., 1986; Aebi et al., 1986, 1988; for a review see Parry and Steinert, 1992). In the case of NF-L, the rod domain is 308-amino acid long and is sandwiched between a 93-amino acid long NH2-terminal head domain and a 142-amino acid long COOH-terminal tail domain (Lewis and Cowan, 1983; Geisler et al., 1985a). The central rod with its periodic distribution of charged residues is thought to be important for the lateral interaction of dimers to form tetramers and possibly higher-order oligomers (e.g., McLachlan and Stewart, 1982; Crewther et al., 1983; for a review see Parry and Steinert, 1992).

Although the head and tail domains flanking the α-helical rod are of variable size and character among different IF subunits (for a review see Steinert and Roop, 1988), two short sequences of positively and negatively charged residues at the COOH- and NH2-terminal ends of the rod reveal a remarkably high conservation among the different types of IF proteins (e.g., Conway and Parry, 1988; Letai et al., 1992). Deletion and point mutations within these two short regions, coupled with transfection and in vitro assembly studies, have indicated that these two sites may play a crucial role in the association of IF dimers into distinct higher-order structures (e.g., Albers and Fuchs, 1987, 1989; Heald and McKeon, 1990; Coulombe et al., 1990; Hatfield and Weber, 1991; Letai et al., 1992). In fact, single-point mutations in these highly conserved rod domain sequences of human epidermal keratins appear to be the proximal cause of two skin blistering diseases (e.g., Bonifas et al., 1991; Coulombe et al., 1991; Lane et al., 1992; Rothnagel et al., 1992; Cheng et al., 1992; Chipev et al., 1992; for recent reviews see Fuchs and Coulombe, 1992; Coulombe, 1993).

Although tetramers do form with only the rod domain (e.g., Geisler et al., 1985b, 1992), a priori it is not evident to what extent the lateral association of two IF dimers into a tetramer is influenced by the presence of the NH2-terminal head and/or COOH-terminal tail domains. On the one hand, electron micrographs of what were assumed to be IF tetramers have been interpreted to represent an approximately in-register alignment of the two dimers within the tetramer in either a parallel (e.g., Ip et al., 1985; Ip, 1988; Quinlan et al., 1984; Hisanaga et al., 1990; Hisanaga and Hirokawa, 1990; Coulombe and Fuchs, 1990) or an antiparallel (Geisler et al., 1985b; Steinert, 1991a) fashion. On the other hand, both EM and chemical cross-linking of tetrameric rods, protofilaments, and assembled IFs have indicated that tetramers are also formed by antiparallel, staggered association of two dimers (e.g., Ip et al., 1985; Potschka et al., 1990; Coulombe and Fuchs, 1990; Steinert, 1991a; Geisler et al., 1992). Possibly unifying these various findings, analysis of the mechanism of in vitro assembly of mouse and human keratins has suggested that IFs are built from multiple oligomeric units rather than a unique tetrameric building block (Coulombe and Fuchs, 1990; Steinert, 1991b). Extending these latter studies, chemical cross-linking has yielded three dominant modes of antiparallel assembly for keratin (Steinert and Parry, 1993; Steinert et al., 1993a,b), vimentin (Steinert et al., 1993c), and desmin (Geisler et al., 1992; Geisler, 1993) dimers: the first involves dimers in approximate register, while in the second dimers are staggered so as to bring the helix 1B segments into approximate alignment, and in the third dimers are staggered so as to bring the helix 2B segments into approximate register.

Whereas it remains to be seen what specific—if any—role(s) the two end domains play in IF tetramer formation, it is without question that assembly of the tetrameric building blocks into higher-order oligomers and ultimately bona fide 10-nm filaments requires at least some parts of one or both end domains to be present. For instance, it has been demonstrated that removal of at least some portions of the NH2-terminal head domain of keratins has deleterious effects to filament formation in vivo and in vitro (e.g., Sauk et al., 1984; Albers and Fuchs, 1989; Coulombe et al., 1990; Bader et al., 1991; Wilson et al., 1992). On the other hand, at least in some (e.g., Hatzfeld and Weber, 1990; Bader et al., 1991; Wilson et al., 1992)—but not all (e.g., Kouklis et al., 1991)—cases, both the type I and type II tail domains of keratins appear to be dispensable for IF assembly. Proteolysis (Geisler et al., 1982; Kaufmann et al., 1985), mutagenesis (Raats et al., 1990, 1991; Eckel et al., 1992; Herrmann et al., 1992; McCormick et al., 1993), and the use of antibodies or synthetic peptides (Birkenberger and Ip, 1990; Hofmann and Herrmann, 1992) have all demonstrated that head or tail domain sequences can markedly affect assembly of desmin and vimentin into IFs. For the nuclear lamins (i.e., type V IF proteins) too, in vitro studies with full length and truncated human, Xenopus and chicken isoforms have shown that both the NH2- and COOH-terminal end domains are implicated in filament formation (Moir et al., 1991; Gieffers and Krohne, 1991; Heitlinger et al., 1992). Taken together, consideration of all of these reports makes it difficult to come to a consensus on the exact contributions of the head and tail domains to IF assembly. Nevertheless, in general the NH2-terminal head domain appears to play a more important role for filament formation than the COOH-terminal tail domain.

For type IV IF proteins specifically, transfection studies with COOH- and NH2-terminal mutants of NF-L and NF-H have already implicated both their head and tail domains in neurofilament formation (Gill et al., 1990; Wong and Cleveland, 1990; Chin et al., 1991). To investigate more systematically the influence of the NH2-terminal head, the central rod, and the COOH-terminal tail domain on the structure and in vitro assembly properties of NF-L, full-length mouse NF-L was expressed in E. coli, purified, and compared with authentic NF-L isolated from bovine spinal cord. In addition, using the same bacterial expression system, recombinant headless, tailless, and rod NF-L fragments were produced, and their structure and assembly properties explored by conventional and scanning transmission electron microscopy (TEM and STEM).

Materials and Methods

(a) Construction of Bacterial Expression Plasmids for Full-length and Truncated Mouse NF-Ls

To facilitate the cloning of full-length and truncated NF-L cDNAs into the bacterial expression vector pET11a, a PCR strategy was employed whereby pairs of synthetic oligonucleotide primers were used to introduce an
Nde I at the translation start codon (ATG) at the 5' end, and a stop codon (TGA) followed by a Bam HI site at the 3' end of each construct (see Fig. 1 a). The parental plasmid pMSV-NF-L cDNA representing the full-length mouse NF-L sequence (Fig. 1 a; see also Gill et al., 1990) was used to prime the synthesis of each of the PCR products encoding NF-L (full-length), NF-L-$N_{A84}$ (nearly headless), NF-L-$N_{A93}$ (exactly headless), NF-L-$C_{A128}$ (nearly tailless), and NF-L-$N_{A84}/C_{A128}$ (rod) (Fig. 1 b). Each of the PCR products was digested with Nde I and Bam HI, gel purified, and ligated to the pET1a expression vector (Novagen, Inc., Madison, WI), previously digested with Nde I and Bam HI, to yield the expression plasmids pET1a-NF-L, pET1a-NF-L-$N_{A84}$, pET1a-NF-L-$N_{A93}$, pET1a-NF-L-$C_{A128}$, and pET1a-NF-L-$N_{A84}/C_{A128}$. The terminal sequences of each construct were verified by the dideoxy-chain termination method.

(b) Bacterial Expression and Purification of Full-length and Truncated Mouse NF-Ls

Each of the PCR products was digested with Nde I and Bam HI, gel purified, and ligated to the pET1a expression vector (Novagen, Inc., Madison, WI), previously digested with Nde I and Bam HI, to yield the expression plasmids pET1a-NF-L, pET1a-NF-L-$N_{A84}$, pET1a-NF-L-$N_{A93}$, pET1a-NF-L-$C_{A128}$, and pET1a-NF-L-$N_{A84}/C_{A128}$. The terminal sequences of each construct were verified by the dideoxy-chain termination method.

Full-length and Truncated Mouse NF-Ls

Each of the PCR products was digested with Nde I and Bam HI, gel purified, and ligated to the pET1a expression vector (Novagen, Inc., Madison, WI), previously digested with Nde I and Bam HI, to yield the expression plasmids pET1a-NF-L, pET1a-NF-L-$N_{A84}$, pET1a-NF-L-$N_{A93}$, pET1a-NF-L-$C_{A128}$, and pET1a-NF-L-$N_{A84}/C_{A128}$. The terminal sequences of each construct were verified by the dideoxy-chain termination method.

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Results

To assess the roles of the 93-amino acid long NH₂-terminal head, 308-amino acid long rod and 142-amino acid long COOH-terminal tail domains in NF-L assembly, a full-length, two 'headless' (NF-L-NΔ84 and NF-L-NΔ93), a 'tailless' (NF-L-CΔ128), and a rod (NF-L-NΔ84/CΔ128) construct were engineered from a mouse NF-L cDNA (see Fig. 1a; Lewis and Cowan, 1985). As described in Materials and Methods, each construct was expressed in bacteria and the recombinant protein purified to better than 95% (see Fig. 2).

(a) Structure and Assembly Properties of Recombinant full-length Mouse NF-L, and Comparison with Authentic Bovine NF-L

As illustrated in Figs. 3 and 4, when dialyzed overnight into standard filament forming buffer (i.e., 50 mM MES, 170 mM NaCl, 1 mM DTT, pH 6.25), recombinant full-length mouse NF-L (Fig. 4) exhibited assembly properties that were very similar to those of authentic NF-L isolated from bovine spinal cord (Fig. 3). In negatively stained preparations both authentic (Fig. 3a) and recombinant (Fig. 4a) NF-L yielded a mixture of “compact” and “partially unraveled” filaments with lengths ranging from 0.5 to several μm. While the more abundant compact, featureless filaments revealed an apparent diameter typical for IFs (i.e., ~10 nm; see Table I), the partially unraveled filaments unveiled the subfilamentous IF organization (c.f., Franke et al., 1982; Aebi et al., 1983, 1988; Troncoso et al., 1990). As documented in Table I, when prepared by glycerol spray-ing/rotary metal shadowing both authentic (Fig. 3b) and recombinant (Fig. 4b) NF-L filaments revealed a 21-nm axial “beading” diagnostic for IFs (Milam and Erickson, 1982; Henderson et al., 1982; Aebi et al., 1983, 1988; Hisanaga and Hirokawa, 1988; Troncoso et al., 1989, 1990; Gotow et al., 1992). Raising the pH of the standard filament forming buffer from its optimal value of 6.25 to the more physiological value of 7.5 produced only short (<100 nm), less than 10-nm wide kinky filamentous assemblies after dialysis of urea-solubilized authentic (Fig. 3c) or recombinant (Fig. 4c) NF-L against otherwise standard filament forming buffer. These short filamentous assemblies appeared rather unstable in the sense that they frequently splayed or unraveled into distinct subfilamentous structures.

Taken together, under standard filament forming conditions the 10-nm filaments assembled from recombinant mouse NF-L appeared indistinguishable from those made of authentic bovine NF-L both by negative staining and glycerol spraying/rotary metal shadowing. Compared to bona fide type I/II or type III IFs, both authentic and recombinant NF-L 10-nm filaments are relatively short and irregular with a clear tendency to partially unravel. The reason for these differences is unclear but may be related to the fact that in vivo neurofilaments are obligate heteropolymers (Lee et al., 1993; Ching and Liem, 1993).

To assess more quantitatively the possible differences be-
Figure 2. Purified authentic bovine NF-L, and the expression and purification of bacterially expressed full-length mouse NF-L, and the mouse NF-L fragments headless, tailless, and rod monitored by SDS-PAGE (10.5%; stained with Coomassie blue). Uninduced (-) and induced (+) bacteria after transformation with the respective expression plasmids (the equivalent of 150 μl of bacterial culture was loaded onto each lane), and (*) purified NF-L or NF-L fragments. (Lane 1) Purified authentic NF-L isolated from bovine spinal cord; (lanes 2–4) bacterially expressed full-length mouse NF-L; (lanes 5–7) bacterially expressed headless mouse NF-L; (lanes 8–10) bacterially expressed tailless mouse NF-L; and (lanes 11–13) bacterially expressed mouse NF-L rod domain. Gel standards with their respective MW, β-galactosidase, 116 kD; phosphorylase b, 97 kD; bovine albumin, 66 kD; and ovalbumin, 43 kD.

(b) Structure and Assembly Properties of Headless Mouse NF-L

To determine the role of the NH2-terminal head domain of NF-L in IF assembly, in vitro reconstitution experiments were performed with NF-L-N484, a truncated mouse NF-L protein missing all but the last nine amino acid residues of its NH2-terminal head domain. As illustrated in Fig. 5a, dialysis of this nearly headless NF-L protein into standard filament forming buffer produced 30–50 nm long rod-like particles with a definite tendency to associate longitudinally when viewed by glycerol spraying/rotary metal shadowing. Most likely, these rod-like particles represent tetramers made of two dimers interacting laterally in an antiparallel fashion with a 20–30 nm overlap, i.e., approximately half-staggered (see Fig. 8a and Discussion). In contrast to tailless NF-L and NF-L rod (see [c] and [d] below), the protofilaments made of headless NF-L did not exhibit any significant tendency to further aggregate laterally in standard filament forming buffer. For lateral aggregation of the protofilaments to ensue, >2 mM CaCl2 or MgCl2 had to be added to standard filament forming buffer (data not shown). Ca2+- or Mg2+-dependent lateral aggregation of protofilaments became even more effective when at the same time the ionic strength (i.e., the NaCl concentration) was lowered.

As documented in Fig. 5, b and c, the headless NF-L construct formed long filamentous structures after overnight dialysis at room temperature of urea-solubilized material into pH 6 MES buffer containing 2–25 mM CaCl2 (see Materials and Methods). These headless NF-L filaments were significantly longer (several μm) and thinner (8.3 ± 0.8 nm; see Table I) than the ~10-nm diameter filaments produced from full-length authentic or recombinant NF-L (compare Fig. 5b with Figs. 3a and 4a). After glycerol spraying/rotary metal shadowing (Fig. 5c), the headless NF-L filaments yielded partially unraveled bundles of protofilaments (compare with Fig. 5a) that apparently lacked the 21-nm axial beading diagnostic for bona fide IFs (see Figs. 3b and 4b) except in places with strong unraveling (see Fig. 5c; arrowheads). In agreement with this finding, when imaged with a scanning force microscope (SFM) in their native buffer environment, headless NF-L filaments adsorbed to glass surfaces also tended to unravel into protofilaments (data not shown). This and the reduced amount of filaments on the EM grid after glycerol spraying/rotary metal shadowing indicated that the lateral interaction of the protofilaments within the headless NF-L filaments was significantly weaker than in the 10-nm filaments produced from full-length NF-L. Interestingly, unlike the 10-nm filaments assembled from full-length NF-L which always yielded some partially unraveled filaments when negatively stained with uranyl acetate or formate (pH 4.0–4.5; see Figs. 3a and 4a), or when imaged by SFM in their native buffer environment (data not shown), headless NF-L filaments did not exhibit any significant unraveling after negative staining with uranyl formate or acetate (Fig. 5b), indicating that these salts stabilized the lateral interaction of the protofilaments within the headless NF-L filaments. In contrast, negative staining of the same headless NF-L filaments with Na-phosphotungstate (pH 7.0) caused complete dissociation of the filaments into protofilaments and small oligomers (data not shown).

To exclude the possibility that filament formation of the
Figure 3. Assembly properties of authentic NF-L isolated from bovine spinal cord. (a, b, and d) Filaments obtained after dialysis of urea-solubilized material against 50 mM MES, 170 mM NaCl, 1 mM DTT, pH 6.25 (standard filament forming buffer) for >12 h at 37°C, or (c) short, kinky filamentous structures formed after dialysis against 50 mM Hapes, 170 mM NaCl, 1 mM DTT, pH 7.5 (“near-physiological” buffer) for >12 h at room temperature. In a and c the samples were negatively stained, in b the material was glycerolsprayed/rotary metal-shadowed, and in d the filaments were fixed with 0.1% glutaraldehyde and freeze-dried unstained for observation in the STEM. STEM MPL data were obtained from 512 × 512-pixel frame low-dose images recorded with a pixel size of 0.9 nm and an average electron dose of 370 ± 44 e-/nm². (e) The MPL values of 593 individual filament stretches were pooled into a histogram which was fitted with a single Gaussian curve to yield an average MPL of 47 ± 7 kDa/nm. Bars, 100 nm (a–d).
"nearly" headless NF-L construct was dependent on the remaining nine amino acid residues of the head domain, an "exact" headless mouse NF-L construct (NF-L-NA93) was engineered (see Fig. 1 b), expressed and purified. Upon dialysis into pH 6.0 MES buffer containing 2-25 mM CaCl₂, long, 8.3-nm wide filaments were obtained (data not shown) that were indistinguishable from those formed from the nearly headless NF-L-NA84 construct (Fig. 5 b).

To quantify the difference between full-length and headless NF-L filaments, we measured their FWHM and MPL by STEM of lightly fixed (with 0.1% glutaraldehyde), unstained specimens (Figs. 3 d and 5 d; see Materials and Methods). As listed in Table I, the headless NF-L filaments (Fig. 5, d and e) yielded an average FWHM of 8.5 ± 1.3 nm and an average MPL of 27 ± 7 kD/nm compared to an average FWHM of 10.1 ± 0.8 nm and an average MPL of 57 ± 6 kD/nm for the recombinant full-length NF-L filaments (Fig. 4, d and e). This dramatic MPL difference cannot simply be explained on the basis of the reduced mass of the headless NF-L fragment, which is ∼55 kD compared to ∼65 kD for the full-length NF-L protein, and would thus yield a MPL of ∼48 kD/nm assuming the same molecular architecture as for the full-length recombinant NF-L filaments, i.e., ∼41 polypeptides per filament cross-section (see [a] above).

Attempts to produce paracrystalline arrays with the headless NF-L fragment were unsuccessful. Whereas filaments with a relatively constant width (e.g., Fig. 5 b) were produced between pH 6 and 7 in the presence of 2-25 mM CaCl₂ or MgCl₂, above pH 7 only short, unstable filamentous particles formed, which upon addition of increasing amounts of divalent cations formed large aggregates (data not shown).

(c) Structure and Assembly Properties of Tailless Mouse NF-L

Under standard filament forming conditions the nearly tailless NF-L, recombinant NF-L-CA128, yielded only small amounts of rod-like particles on EM grids both by negative staining as well as after glycerol spraying/rotary metal shadowing (data not shown). Judged from the turbidity of the assembled material, it consisted of large structures—possibly aggregates of 10-nm filaments—that only poorly adsorbed to EM grids. In contrast, in the presence of mM amounts of CaCl₂, the tailless NF-L polypeptide assembled into filamentous and paracrystal-like structures in the pH range 6-7.5 after dialysis for ≥3 h into NaCl-free buffer at room temperature. As illustrated in Fig. 6, a and b, with 1 mM Ca²⁺ tailless NF-L formed long filaments which at pH 7.5 exhibited a strong tendency to associate laterally into filament bundles. Like the headless filaments, the tailless NF-L filaments revealed a molecular architecture distinct from that of the typical 10-nm filaments formed with full-length NF-L: (a) they yielded a larger apparent width in negatively stained preparations, i.e., 11.3 nm vs 10.4 nm (see Table I), and (b) they did not exhibit the 21-nm axial beading characteristic for bona fide IFs after glycerol spraying/rotary metal shadowing (compare Fig. 6 b with Figs. 3 b and 4 b). As a significant amount of the assembled material was too big to adsorb to EM grids, it was plastic-embedded and thin-sectioned (see Materials and Methods) for visualization. As documented in Fig. 6 c, just as the smaller structures captured in negatively stained (Fig. 6 a) or glycerol-sprayed/rotary metal shadowed (Fig. 6 b) preparations, the large assembly products consisted of bundles of tightly packed filaments.

Raising the Ca²⁺-concentration from 1 to 10 mM at pH 7.5 led to a significant increase of the filament width. As illustrated in Fig. 6 d, this condition produced paracrystalline fibers exhibiting a distinct light-dark transverse banding pattern with an approximate axial periodicity of 20.8 ± 1.1 nm (see Table I). Under these conditions too, a significant amount of large aggregates formed that, when embedded and thin-sectioned, revealed similar paracrystalline structures (Fig. 6 e) with an approximate axial periodicity of 20.4 ± 0.9 nm (see Table I) as were obtained after negative staining (Fig. 6 d). As documented in Fig. 6 f, raising the CaCl₂-concentration to 20 mM induced formation of large, three-dimensional paracrystalline arrays with the same light-dark transverse banding pattern and an approximate axial periodicity of 21.2 ± 0.6 nm (see Table I). Substitution of Mg²⁺ for Ca²⁺ led to similar three-dimensional paracrystalline arrays with an approximate axial periodicity of 21 nm. Whereas all the paracrystalline arrays obtained with tailless NF-L (Fig. 6, d-f) yielded a light-dark transverse banding pattern with a fairly regular axial periodicity of ∼21 nm (see Table I), their true axial repeat was probably more like 42 nm (see Fig. 9 b and Discussion).

(d) Structure and Assembly Properties of the Mouse NF-L Rod Domain

Unlike the full-length (Fig. 4, a and b), headless (Fig. 5, b and c), and tailless (Fig. 6, a and b) mouse NF-L constructs, the recombinant mouse NF-L rod domain failed to form filamentous structures under all conditions examined. Just like the tailless NF-L fragment (see [c] above), under standard filament forming conditions the NF-L rod domain produced large aggregates that did not stick to EM grids. While these aggregates may actually consist predominantly of small, distinct oligomers (e.g., tetramers or octamers), this behavior is very different from that of desmin and vimentin rods and the rods made of keratin pairs which form soluble tetramers under typical filament forming conditions (e.g., Sauk et al., 1984; Geisler et al., 1992). So it could be argued that this atypical behavior of the NF-L rod at pH 6.25 may simply represent "isoelectric precipitation". Arguing against this interpretation, formation of similar aggregates that also failed to adsorb to EM grids was observed after dialysis of the NF-L rod against near-physiological buffer (i.e., 50 mM Hepes, 170 mM NaCl, 1 mM DTT, pH 7.5; data not shown).

Another possibility for this unusual behavior of the NF-L rod is that this atypical behavior of the NF-L rod at pH 6.25 may simply represent "isoelectric precipitation". Arguing against this interpretation, formation of similar aggregates that also failed to adsorb to EM grids was observed after dialysis of the NF-L rod against physiological buffer (i.e., 50 mM Hepes, 170 mM NaCl, 1 mM DTT, pH 7.5; data not shown). Another possibility for this unusual behavior of the NF-L NA84/CA128 rod used in this investigation may be its lack of a several residue long consensus sequence found in all IF proteins (e.g., Steinert and Roop, 1988; Steinert and Parry, 1993) which was still present in, e.g., the proteolytically produced desmin rod forming soluble tetramers at physiological salt and pH conditions (e.g., Geisler et al., 1992).

In the presence of >20 mM divalent cations (i.e., CaCl₂ or MgCl₂), the NF-L rod assembled into large, precipitating aggregates after dialysis into pH 7.0 MES buffer. As documented in Fig. 7 a, the smaller aggregates that adsorbed to EM grids appeared as highly branched supramolecular assemblies in negatively stained preparations. Closer inspec-
Figure 4. Assembly properties of recombinant mouse NF-L. (a, b, and d) Filaments obtained after dialysis of bacterially expressed, ureasolubilized mouse NF-L against 50 mM MES, 170 mM NaCl, 1 mM DTT, pH 6.25 (standard filament forming buffer) for ≥12 h at 37°C, or (c) short, kinky filamentous structures formed after dialysis against 50 mM Hepes, 170 mM NaCl, 1 mM DTT, pH 7.5 ("near-physiological" buffer) for ≥12 h at room temperature. In a and c the samples were negatively stained, in b the material was glycerol-
tion of the thin edges of these assemblies revealed faint, light-dark transverse banding patterns (Fig. 7 a; arrowheads) with an approximate axial periodicity of 21.4 ± 1.4 nm (see Table I), indicating that they were paracrystalline in nature. Similar to the tailless NF-L fragment (see Fig. 6 a), when embedded and thin-sectioned, the aggregates and precipitates produced by the NF-L rod domain under these assembly conditions were made of paracrystalline fibers or "needles" exhibiting a distinct light-dark transverse banding pattern with an approximate axial periodicity of 20.7 ± 1.6 nm (see Table I). However, unlike the tailless NF-L fragment, negative stain electron microscopy of the NF-L rod domain revealed no filamentous structures or fibers at any Ca²⁺- or Mg²⁺-concentration tried. On the other hand, the conditions under which tailless NF-L and the NF-L rod formed paracrystalline arrays were very similar (see [c] above).

**Table I. Various Measurements of Distinct Assembly Products of Full-length Authentic and Recombinant NL-L, and of Recombinant Headless, Tailless, and Rod Fragments of NF-L**

| NF-L construct | Width of filaments (nm) | Mass-per-length (MPL) of filaments (kD/nm) | Approximate axial periodicity (nm) |
|----------------|-------------------------|-------------------------------------------|----------------------------------|
| full-length NL-L (authentic) | 10.2 ± 0.8 (n = 100)* | 47 ± 7 (n = 393)I | 21.2 ± 0.6 (n = 390)I |
| full-length NL-L (recombinant) | 10.4 ± 1.0 (n = 100)* | 57 ± 6 (n = 507)I | 21.3 ± 0.4 (n = 449)I |
| headless NF-L | 8.4 ± 0.8 (n = 100)* | 27 ± 7 (n = 277)I | - |
| tailless NF-L +1 mM CaCl₂ | 11.3 ± 1.7 (n = 100)* | not measured | 20.8 ± 1.1* |
| +10 mM CaCl₂ | no filaments | - | 20.4 ± 0.9I |
| +20 mM CaCl₂ | no filaments | - | 21.2 ± 0.6* |
| NF-L rod | no filaments | - | 21.4 ± 1.4* |

The mean ± SD have been calculated for each set of n measurements.
* Negatively stained (compact filaments only were measured)
† Glycerol-sprayed/rotary metal-shadowed
‡ Embedded/thin-sectioned
§ Unstained (fixed with 0.1% glutaraldehyde) filaments recorded in a STEM; in the case of the filament width, the FWHM was determined (for the method see Engel et al., 1985).

From these images it is evident that the appearance of beaded segments with an average, 21-nm axial repeat does not require an entire 10-nm filament. In fact, as documented in Fig. 8 b (arrowheads) and shown previously with keratins (Sauk et al., 1984; Coulombe and Fuchs, 1990), it is already night (Hisanaga et al., 1990) yielded small oligomeric particles consisting of 3–5 beaded segments with an average axial repeat of 21 nm (see arrowheads). Most likely, these particles represent octamers (Hisanaga et al., 1990), i.e., pairs of approximately half-staggered tetramers. Based on these data it is difficult to decide whether the tetramers themselves have a stagger or not (e.g., Aebi et al., 1988; Coulombe and Fuchs, 1990; Steinert, 1991b). Similarly, as illustrated in Fig. 8 b, unraveling NL-L filaments (i.e., in the presence of 10 mM phosphate; e.g., Aebi et al., 1983, 1988; Troncoso et al., 1990) unveiled subfilaments also built from beaded segments with an average axial repeat of 21 nm. Probably, these subfilaments are primarily octameric prototibrils (Aebi et al., 1983) with the more massive ones being pairs of prototibrils. Occasionally, rather tenuous subfilaments can be depicted (see arrowheads) which most likely represent tetrameric prototibrils. Interestingly, in several instances the 21-nm beading repeat along these prototibrils or prototibrils appears irregular, alternating between shorter and longer repeats (see also Sauk et al., 1984; for a possible explanation see Fig. 9 b and Discussion). For comparison, Fig. 8 c reveals a stretch of intact NL-L filament which in places appears split into two "half-filaments" (see arrow) probably consisting of pairs of prototibrils (see Fig. 9 b and Discussion).

From these images it is evident that the appearance of beaded segments with an average, 21-nm axial repeat does not require an entire 10-nm filament. In fact, as documented in Fig. 8 b (arrowheads) and shown previously with keratins (Sauk et al., 1984; Coulombe and Fuchs, 1990), it is already
observed with individual tetrameric protofilaments, albeit with smaller beads and less regular repeats. Most likely, a significant contribution to the appearance of beads is made by the NH₂- and COOH-terminal end domains, as their removal attenuates or abolishes the beading (see Figs. 5 c and 6 b). An additional contribution must involve the longitudinal, head-to-tail interaction of dimers with a 1-3-nm N-C type overlap thus producing “bumps” (see Fig. 9 and Discussion). Finally, the average 21-nm axial periodicity of the beaded segments is due to an approximate half-stagger between laterally associating dimers, tetramers, or octamers (for details see Fig. 9 and Discussion).

Discussion

(a) Toward a Molecular Model for IF Architecture

How can EM and chemical cross-linking data be combined to yield a molecular model for IF architecture? The elementary building block of all IFs is the dimer formed by a parallel, unstaggered two-stranded α-helical coiled-coil with a molecular length ranging between 44 and 46 nm (e.g., Geisler et al., 1982; Hanukoglu and Fuchs, 1982, 1983; Steinert et al., 1983; Parry et al., 1985; Quinlan et al., 1986; Aebi et al., 1986, 1988; Steinert et al., 1993a,c). As illustrated in Fig. 9 a, at their first level of structural organization dimers interact laterally, either unstaggered or approximately half-staggered, to form tetramers (e.g., Sank et al., 1984; Ip et al., 1985; Geisler et al., 1985b, 1992; Coulombe and Fuchs, 1990; Steinert, 1991b; Steinert et al., 1993a,c). In contrast, the nuclear laminas with a ~45-kD central rod domain (e.g., McKeon et al., 1986; Fisher et al., 1986) amounting to a dimer length of 50–52 nm (e.g., Aebi et al., 1986; Moir et al., 1991; Heitlinger et al., 1991, 1992), have revealed a longitudinal, head-to-tail interaction of dimers with a 1-3-nm N-C type overlap (see Fig. 9 a; and Gielfers and Krohne, 1991; Heitlinger et al., 1991, 1992).

More specifically, with regard to molecular models for NF-L filament architecture, the approximate, 21-nm axial repeat of beaded segments (see Table I) observed with filaments of both authentic (Fig. 3 b) and recombinant (Fig. 4 b) NF-L after glycerol spraying/rotary metal shadowing, and the approximate, 21-nm axial periodicity (see Table I) seen in paracrystals made of tailless NF-L (Fig. 6, d-f) and NF-L rod (Fig. 7) impose two constraints: (a) As the true axial repeat of either filaments or paracrystals is 42–43 nm (i.e., 2 × 21–21.5 nm; see Table I) and hence shorter than the molecular length l of the dimer building blocks (see Fig. 9 a), which for NF-L is likely to be somewhere between 44 and 45 nm (e.g., Steinert et al., 1993a,c; Parry, D. A. D., personal communication), their longitudinal association must involve a 1-3-nm head-to-tail “overlap.” (b) The approximate, 21-nm axial periodicity found in both filaments and paracrystals must involve an approximate “half-stagger” between at least some of the dimers when they associate laterally to form higher-order oligomers.

In Fig. 9 b an attempt has been made to use these two constraints to design packing models for protofilaments, protofibrils, and half-filaments, and to deduce the “periodicity patterns” they produce, i.e., as manifested by the axial beading observed with filaments, or the transverse banding patterns seen with paracrystalline arrays. It should be stressed, however, that the hierarchy of the three types of lateral dimer–dimer interactions (Fig. 9 a) involved—the occurrence of all of which has been documented by chemical cross-linking (e.g., Geisler et al., 1992; Geisler, 1993; Steinert et al., 1993a,b,c)—is ambiguous. In these packing models, the approximate, 21-nm axial periodicity represents the average of the two axial periodicities r₁ and r₂, which are determined by the exact stagger s respectively overlap o between dimers (see Fig. 9 a), with r₁ + r₂ = r being the true axial repeat (see Fig. 9 b). What implications does the proposed NF-L dimer packing model which, conceptually, is very similar to the IF model proposed recently based on keratin and vimentin cross-linking data (Steinert et al., 1993a,c) have for IF structure and assembly in general?

In both models the longitudinal association of dimers involves a short head-to-tail overlap (see Fig. 9 a): in our model it is somewhere between 1 and 3 nm (see above), whereas in the Steinert and Parry model it is 1.01 nm for keratin (i.e., with a molecular length of 46.22 nm) and 1.2 nm for vimentin (i.e., with a molecular length of 43.91 nm) IFs (Steinert et al., 1993c). This overlap may explain the remarkably high conservation of short sequences of positively and negatively charged residues found among different types of IF proteins (e.g., Conway and Parry, 1988; Letai et al., 1992). Hence, it is not surprising that deletions and point mutations within these two short end segments have profound effects on the association of IF dimers into distinct higher-order structures (e.g., Albers and Fuchs, 1987, 1989; Gill et al., 1990; Wong and Cleveland, 1990; Coulombe et al., 1990; Hatzfeld and Weber, 1991; Letai et al., 1992), and in several cases appear to be the proximal cause of genetic skin diseases of keratin (for review see Fuchs and Coulombe, 1992; Coulombe, 1993).

As suggested by the packing models in Fig. 9 b and documented with NF-L data in Fig. 8 (see also Sank et al., 1984; Ip et al., 1985; Coulombe and Fuchs, 1990), contrary to the “surface lattice” model (e.g., Steinert et al., 1993a), observation of the approximate, 21-nm axial periodicity does

Figure 5. Assembly properties of bacterially expressed headless mouse NF-L. (a) Protofilamentous structures produced after dialysis of urea-solubilized headless mouse NF-L against 50 mM MES, 170 mM NaCl, 1 mM DTT, pH 6.25 (standard filament forming buffer) for >12 h at 37°C. Short protofilaments together with protofilamentous particles predominate. (b–d) Filaments obtained after dialysis of urea-solubilized headless mouse NF-L against 50 mM MES, 1 mM DTT, pH 6.0, containing 6 mM CaCl₂ for >12 h at room temperature. In a and c the samples were glycerol-sprayed/rotary metal-shadowed (arrowheads in c point to ~21-nm axial beading repeats), in b the material was negatively stained, and in d the filaments were fixed with 0.1% glutaraldehyde and freeze-dried unstained for observation in the STEM. STEM MPL data were obtained from 512 × 512-pixel frame low-dose images recorded with a pixel size of 0.9 nm and with an average electron dose of 318 ± 32 e⁻/nm². (e) The MPL values of 277 individual filament stretches were pooled into a histogram which was fitted with a single Gaussian curve to yield an average MPL of 27 ± 7 kD/nm. Bars, 100 nm (a–d).
not require an entire 10-nm filament but becomes already manifest with tetrameric protofilaments or octameric protofibrils, provided they involve an N-N or C-C type approximate half-stagger between dimers. In fact, exactly as the periodicity patterns of the tetramer and octamer packing models predict (see Fig. 9 b), in some instances alternating shorter (i.e., \( r_1 \)) and longer (i.e., \( r_2 \)) axial repeats can be distinguished along protofilaments or protofibrils (see Results and Fig. 8, a and b).

Whereas the short head-to-tail overlap of dimers gives rise to "bumps" which are accentuated by the presence of the end domains, combination of this longitudinal N-C type overlap with either one of the approximately half-staggered N-N or C-C type lateral interactions of dimers generates "gaps" in the three-dimensional packing of dimers into higher-order oligomers. These bumps and gaps follow the approximate, 21-nm axial periodicity pattern and thereby produce the beaded segments and transverse banding patterns seen in filaments and paracrystalline arrays, respectively.

It must be emphasized that all molecular models for IF structure must be viewed in the framework of an ambiguity of what a bona fide 10-nm filament actually is. MPL measurements using quantitative STEM have provided strong evidence that at least in vitro assembled IFs are polymorphic in the sense that their number of polypeptides per filament cross-section varies both between different as well as along the same filaments (for review see Aebi et al., 1988). Unlike that of actin filaments or microtubules, the IF architecture may not be uniquely specified. This ambiguity may be most pronounced for neurofilaments since despite in vitro assembly of NF-L into 10-nm filaments, in vivo neurofilaments are obligate heteropolymers (Lee et al., 1993; Ching and Liem, 1999).

Figure 6. Assembly properties of bacterially expressed tailless mouse NF-L. (a–c) Filament bundles obtained after dialysis of urea-solubilized tailless mouse NF-L against 50 mM Hepes, 1 mM DTT, pH 7.5, containing 1 mM CaCl\(_2\) for >3 h at room temperature. (d and e) Paracrystalline fibers with an approximate axial periodicity of 20.8 ± 1.1 nm (see Table I) yielded after dialysis of urea-solubilized material against 50 mM Hepes, 1 mM DTT, pH 7.5, containing 10 mM CaCl\(_2\) for >3 h. (f) Paracrystalline arrays with an approximate axial periodicity of 21.2 ± 0.6 nm (see Table I) obtained after dialysis of urea-solubilized material against 50 mM Hepes, 1 mM DTT, pH 7.5, 20 mM CaCl\(_2\) for >3 h. Specimens were prepared by either negative staining (a, d, and f), glycerol spraying/rotary metal-shadowing (b) or plastic embedding/thin sectioning (c and e). Bars, 100 nm (a–f).
How substoichiometric amounts (i.e., <10%) of NF-M or NF-H are incorporated into a neurofilament lattice and thereby affect the filament network remains elusive.

(b) The Roles of the NF-L Head and Tail Domains in 10-nm Filament Assembly

Whereas the headless NF-L fragment yielded 30–50-nm long rod-like particles with a tendency to associate longitudinally into protofilament-like structures (see Fig. 5a) under standard filament forming conditions, the tailless NF-L fragment formed predominantly large structures—possibly aggregates of 10-nm filaments—that adsorbed very poorly to EM grids. Similar to tailless NF-L, the NF-L rod domain also produced large aggregates. While definitely not representing aggregates of 10-nm filaments, most likely, they were made of small, distinct oligomers. In contrast, the rod domains of keratins, desmin, and vimentin form soluble oligomers (e.g., tetramers or octamers) under typical filament forming conditions (e.g., Sank et al., 1984; Geisler et al., 1992). As formation of similar aggregates was observed after dialysis of the NF-L rod against near-physiological buffer (i.e., at pH 7.5; data not shown), this atypical behavior cannot simply represent isoelectric precipitation of the NF-L rod at pH 6.25.

Upon appropriate adjustment of the assembly conditions (i.e., inclusion of mM amounts of Ca$^{2+}$ or Mg$^{2+}$) both the headless and tailless NF-L fragments produced filaments, although their morphologies were distinct from that of the 10-nm filaments produced with full-length NF-L. When negatively stained, the filaments formed by headless NF-L appeared thinner (see Table I) and longer than full-length NF-L filaments, whereas after glycerol spraying/rotary metal shadowing they yielded partially unraveled bundles of protofilaments that apparently lacked the 21-nm axial beading diagnostic for bona fide IFs. STEM revealed a MPL corresponding to only 24 truncated—compared to 41 full-length—polypeptides per filament cross-section. In contrast, the negatively stained filaments produced by tailless NF-L appeared wider (see Table I) and more compact than full-length NF-L filaments. In addition, they exhibited a strong tendency to aggregate laterally into bundles. These filaments too lacked the 21-nm axial beading when glycerol-sprayed/rotary metal-shadowed; however, unlike the headless NF-L filaments, they did not unravel into their protofilaments indicating strong lateral interaction. Based on EM and analytical ultracentrifugation in 3–6 M urea, very much like full-length NF-L, the headless, tailless, and rod fragments were competent to form dimers and tetramers (data not shown). Whereas chemical cross-linking has indicated that with desmin the stagger and polarity between dimers is conserved within tetrameric rods, protofilaments and 10-nm filaments (Geisler et al., 1992), it remains to be determined whether this relationship is also conserved among the full-length, headless, tailless, and rod fragments of NF-L.

Taken together, the following roles can be assigned to the two end domains of NF-L during 10-nm filament formation: (a) The NH$_2$-terminal head domain promotes lateral association of protofilaments into protofibrils, and ultimately 10-nm filaments, as its removal terminates assembly at the protofilament level. (b) In contrast, the COOH-terminal tail domain controls lateral assembly of protofilaments so that it
Figure 9. Schematic representation of a molecular model for IF architecture. (a) The elementary building block is the dimer rod which consists of two \(~40\) kD rod domains forming a parallel, untaggered two-stranded \(\alpha\)-helical coiled-coil (i.e., via coils 1A, 1B, 2A, and 2B) with molecular length \(l\). For simplicity, the two end domains have been omitted. Longitudinally, dimers interact head-to-tail with a short, N-C type overlap \(o_{nc}\), thus yielding an axial repeat \(r = l - o_{nc}\). Laterally, dimers may interact in three modes to form antiparallel tetramers (e.g., Steinert et al., 1993a): (1) in approximate register (i.e., untaggered) with an NC-CN type overlap; (2) approximately half-staggered with an N-N type overlap \(o_{nn}\) so as to bring coils 1B into approximate alignment; (3) approximately half-staggered with a C-C type overlap \(o_{cc}\) so as to bring coils 2B into approximate alignment. Based on chemical cross-linking data, the overlaps for the different modes of dimer–dimer interactions have been calculated for keratin and vimentin IFs (Steinert et al., 1993a, b), and for some of those occurring with desmin (Geisler et al., 1992; Geisler, 1993). (b) One possible design of protofilaments, protofibrils, and half-filaments sequentially involving the three possible modes of lateral dimer–dimer interactions together with the longitudinal head-to-tail interaction of dimers as defined in \(a\). In each case, the "periodicity pattern," i.e., as defined by the N-C type overlap pattern, has also been drawn. Together with the end domains this pattern forms the basis for the 21-nm axial beading seen in filamentous NF-L assemblies (see Fig. 8) as well as the distinct light-dark transverse banding patterns with an approximate, 21-nm axial periodicity depicted in paracrystalline arrays made of truncated NF-Ls (see Fig. 6, d–f, and Fig. 7). The choice of the N-N type lateral association of dimers to form the protofilament has been arbitrary, and so has been the combination of N-N and C-C type lateral interactions to produce the protofibril. This becomes evident when trying to delineate protofilaments or protofibrils in the half-filament: there are obviously several possibilities to achieve this.

terminates at the 10-nm filament level, as its removal causes uncontrolled lateral growth and/or aggregation of 10-nm filaments. Hence, the two end domains of NF-L have antagonistic effects on the lateral association of protofilaments into higher-order structures, with the effect of the COOH-terminal tail domain being dominant over that of the NH\(_2\)-terminal head domain. However, as neurofilaments are obligate heteropolymers in vivo (Lee et al., 1993; Ching and Liem, 1993), the actual roles of the NF-L head and tail domains in neurofilament assembly and network formation may be more subtle, as these may be modulated significantly by the presence of NF-M and NF-H.

(c) Differences between Recombinant and Authentic NF-L Filaments May Be Due to In Vivo Glycosylation within the NH\(_2\)-Terminal Head Domain

Although by standard EM the filaments obtained with
recombinant mouse NF-L appeared indistinguishable from those assembled from authentic bovine NF-L, quantitative STEM revealed their MPLs to differ by roughly eight poly-peptides per filament cross-section (see Table I), which as outlined schematically in Fig. 9b corresponds to one octameric protofibril or two tetrameric protofilaments (Eichner et al., 1985; Engel et al., 1985; Aebi et al., 1988; Troncoso et al., 1989). Whereas it is conceivable that this reflects inherent assembly differences between the bovine and mouse NF-L subunits, a more plausible explanation for these assembly differences may be the recent identification of O-glycosidically linked N-acetylglucosamine (O-GlcNAc) monosaccharides to Thr18 and Ser17 at the NH2-terminal head domain of NF-L (Dong et al., 1993). These cytoplasmic glycosylations, which are probably reversible in vivo (Kearse and Hart, 1991), occur at a stoichiometry of ~0.1 mole of GlcNAc per mole of authentic NF-L purified from mouse spinal cord. Since bacteria are not known to have comparable glycosylation enzymes, and since in vitro reconstitution experiments with authentic bovine NF-L have shown that the filament morphology, i.e., length and width, critically depends on the assembly conditions (e.g., pH, ionic strength, divalent cations) employed (Aebi et al., 1988; Troncoso et al., 1989), the observed MPL difference between the recombinant and authentic NF-L filaments is most likely due, at least in part, to these glycosidic modifications of the authentic NF-L head domain.

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