Assembly of Type IV Neuronal Intermediate Filaments in Nonneuronal Cells in the Absence of Preexisting Cytoplasmic Intermediate Filaments

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Abstract. We report here on the in vivo assembly of α-internexin, a type IV neuronal intermediate filament protein, in transfected cultured cells, comparing its assembly properties with those of the neurofilament triplet proteins (NF-L, NF-M, and NF-H). Like the neurofilament triplet proteins, α-internexin coassembles with vimentin into filaments. To study the assembly characteristics of these proteins in the absence of a preexisting filament network, transient transfection experiments were performed with a non-neuronal cell line lacking cytoplasmic intermediate filaments. The results showed that only α-internexin was able to self-assemble into extensive filamentous networks. In contrast, the neurofilament triplet proteins were incapable of homopolymeric assembly into filamentous arrays in vivo. NF-L coassembled with either NF-M or NF-H into filamentous structures in the transfected cells, but NF-M could not form filaments with NF-H. α-internexin could coassemble with each of the neurofilament triplet proteins in the transfected cells to form filaments.

When all but 2 and 10 amino acid residues were removed from the tail domains of NF-L and NF-M, respectively, the resulting NF-L and NF-M deletion mutants retained the ability to coassemble with α-internexin into filamentous networks. These mutants were also capable of forming filaments with other wild-type neurofilament triplet protein subunits. These results suggest that the tail domains of NF-L and NF-M are dispensable for normal coassembly of each of these proteins with other type IV intermediate filament proteins to form filaments.

α-internexin and the neurofilament triplet proteins (designated NF-L, NF-M, and NF-H for low, middle, and high molecular weight subunits, respectively) are members of the type IV intermediate filament (IF)[1] family of proteins (for reviews see Steinert and Roop, 1988; Fliegner and Liem, 1991; Shaw, 1991). α-internexin is expressed abundantly in young, postmitotic neurons of the developing peripheral and central nervous systems, but in the adult it is found primarily in the central nervous system (Chiu et al., 1989; Kaplan et al., 1990). The neurofilament triplet proteins (NFTPs), whose expression is preceded by that of α-internexin, are expressed in mature neurons of both the peripheral and central nervous systems. While all of these type IV IF proteins appear to be present in nearly all mature neurons of the central nervous system, α-internexin is expressed at much lower levels than the NFTPs in large neurons such as the motor neurons of the spinal cord and cranial nerve ganglia (Kaplan et al., 1990). Conversely, α-internexin is highly expressed in

the parallel fibers of the cerebellar granule cells, where the NFTPs are noticeably absent (Kaplan et al., 1990).

α-internexin and the NFTPs conform to a structure typical of all IF proteins, which consists of a highly conserved α-helical rod domain flanked by variable amino-terminal head and carboxyl-terminal tail domains (Steinert and Roop, 1988). The α-helical rod domain contains hydrophobic heptad repeats which are believed to mediate the formation of coiled-coil dimers, the first step in the process of IF assembly. Although the head and tail domains of these proteins show only limited sequence homology, they share certain similarities. The head domains are highly basic, rich in arginine, glycine, and proline, and contain many serine residues, some of which have been found to be the targets of various kinases both in vitro and in vivo (Toru-Delbauffe et al., 1986; Sihag et al., 1988; Sihag and Nixon, 1989). The tail domains contain several sequence motifs (Shaw, 1991): the glutamic acid-rich E region present in all type IV IF proteins; the conserved KE sequence, rich in lysine and glutamic acid residues, and present in α-internexin and NF-M; and finally, the KSP (lysine-serine-proline) and SP (serine-proline) sequences present exclusively in NF-M and NF-H, where they occur multiple times and serve as phosphorylation sites.

In vitro reassembly studies of purified NFTPs showed that

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1. *Abbreviations used in this paper:* IF, intermediate filament; NFTPs, neurofilament triplet proteins.

NF-L could easily polymerize into 10-nm filaments, whereas NF-M and NF-H required the presence of NF-L to copolymerize into filaments of normal morphology (Geisler and Weber, 1981; Liem and Hutchison, 1982). Purified α-internexin was also shown to be able to polymerize into filaments in vitro (Chiu et al., 1989; Kaplan et al., 1990). However, it has not yet been examined whether these proteins display the same kinds of assembly characteristics in vivo.

Transfection studies have shown that the NFTPs could each coassemble into the endogenous vimentin network in fibroblast cell lines (Chin and Liem, 1989, 1990; Monteiro and Cleveland, 1989). Recently, it has been shown by transient transfections that mutants of NF-L and NF-M with substantial portions of the tail domains deleted, as well as a headless NF-L mutant still retained the ability to coassemble into the endogenous vimentin network in fibroblasts (Gill et al., 1990; Wong and Cleveland, 1990; Chin et al., 1991). However, deletions into the α-helical rod domains of either protein resulted in mutants which were unable to coassemble with the endogenous vimentin. While the head domain and most of the tail domain appear to be dispensable, at least for NF-L and NF-M, for normal coassembly with vimentin, a type III IF, the role of these domains in coassembly with other type IV IF proteins in vivo remains to be established. A similar analysis of the coassembly of α-internexin with the type III and IV IF proteins has not yet been reported.

The present work describes the self- and coassembly of transfected type IV IF proteins in a cell line lacking an endogenous cytoplasmic IF network. We also demonstrate by deletion analysis that the variable carboxy-terminal tail domains of NF-L and NF-M are dispensable for their coassembly with the other type IV IF proteins.

Materials and Methods

Construction of Plasmids

The cDNA for rat α-internexin encoding the full-length polypeptide has previously been cloned as a 1.6-kb EcoRI insert into pGEM 7zf(+) (Fliegner et al., 1990). To construct pRSV-α, the 1.6-kb EcoRI fragment was isolated and its ends were filled in with Klenow enzyme and attached to HindIII linkers (CAAGCTTG). After HindIII digestion, the resulting 1.6-kb HindIII fragment was cloned into the HindIII site on the pRSVi-HindIII vector, which contains the Rous sarcoma virus long terminal repeat (Forman et al., 1988). To prepare a promoterless vector for the construction of pαP-α, a PvuII-HindIII fragment containing the SV40 promoter was removed from the pSVi-HindIII vector (Forman et al., 1988) and the PvuII site remaining on the vector was changed to a HindIII site with attachment of HindIII linkers (CAAGCTTG), yielding a promoterless pSV2 (ΔPvuII) vector. To construct pαP-α, a 1.7-kb HindIII-XhoI fragment containing 1.2 kb of the 5' flanking sequence and 436 bp of the 5' coding sequence of the α-internexin gene was isolated from pαEX1.7 (Ching and Liem, 1991), and a 1.2-kb XhoI-HindIII fragment containing the α-internexin cDNA sequence downstream of its unique XhoI site was isolated from pRSV-α, followed by ligation of these two fragments together with the HindIII-digested, promoterless pSV2(ΔPvuII). For construction of M1-421, a 1.3-kb HindIII-SfaNI fragment encoding amino acid residues 1-421 of rat NF-M was isolated from pRSVi-NFM1(+) (Chin and Liem, 1989), and its ends were filled in with Klenow and attached to HindIII linkers (CAAGCTTG). After digestion with HindIII, the resulting 1.3-kb HindIII fragment was cloned into the HindIII site on pRSVi-HindIII. The other constructs, pRSVi-NFH1(+), pRSVi-NFL1(+), and L1-402 have been previously described (Chin and Liem, 1989, 1990; Chin et al., 1991). pRSV-Vim contains the fully-encoding rat vimentin cDNA cloned into the pRSVi-HindIII vector (Chen, W. J., and R. K. H. Liem, unpublished observations). DNA sequencing was performed on the deletion mutant constructs in order to deduce the amino acid sequences at the deletion junctions.

Cell Culture and Transfection

Mouse Ltk⁻ cells were grown as previously described (Ching and Liem, 1991). Human adrenal carcinoma SW13cl.2Vim⁻ (Sarria et al., 1990; a generous gift from Dr. Robert Evans, University of Colorado, Denver, CO) were grown in DMEM/F12 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 5% FBS at 37°C in a humidified atmosphere of 7% CO_2.

For immunofluorescence studies, cells were grown on sterile, untreated glass coverslips 24 h before transfection. Twenty μg of a tested DNA construct (or 10 μg each when two DNA constructs were used) were introduced into cells by calcium phosphate-mediated transfection essentially as previously described (Ching and Liem, 1991). 4 h after transfection, cells were treated with DMEM/F12 medium containing 15% DMSO at room temperature for 2 min, washed, and refed with fresh growth medium. Cells were harvested 48 h after transient transfection. For stable transfection, Ltk⁻ cells that were transfected with 19 μg of pRSV-α (or pαP-α) and 1 μg of pSV2-neo (Southern and Berg, 1982) as described above were subcultured 48 h after transfection, and were subsequently selected and maintained in growth medium containing 400 μg/ml of G418 (Geneticin, GIBCO BRL).

Antibodies

A mouse monoclonal antibody to α-internexin, as well as rabbit polyclonal antibodies to NF-L and NF-M (designated AbNFL$_n$ and AbNFM$_n$), which specifically recognize the amino-termini of rat NF-L and NF-M, respectively, have been previously described (Kaplan et al., 1991). A rabbit polyclonal antibody to α-internexin was prepared against a fusion protein containing the amino-terminal 340 amino acid residues of rat α-internexin, expressed from the pET vector (Kaplan, M. P., and R. K. H. Liem, unpublished results). Rabbit polyclonal anti-NF-L and anti-NF-M antibodies were obtained commercially (Chemicon International, Temecula, CA), as were mouse monoclonals to NF-M (Boehringer Mannheim, Indianapolis, IN) and NF-H (Sigma Chem. Co., St. Louis, MO). A mouse monoclonal anti-NF-L, in the form of ascites fluid, was a gift from Dr. Gerry Shaw (University of Florida, Gainesville, FL). Like most antibodies to the NFTPs, these last five are all directed against epitopes in the tail region. A mouse monoclonal antibody to vimentin was obtained commercially (Sigma Chem. Co.); a rabbit polyclonal anti-vimentin antibody was a gift from Dr. Eugenia Wang (Lady Davis Institute for Medical Research, Montreal).

Indirect Immunofluorescence Staining

Cells grown on coverslips were rinsed in PBS-def (phosphate-buffered saline deficient in Ca^{2+} and Mg^{2+}) and fixed in cold methanol at -20°C for 10 min. After several washes with PBS-def, the cells were treated with PBS-def containing 3% normal goat serum for 30 min and were subsequently washed and incubated with primary antibodies at room temperature for 1 h. The cells were then washed several times with PBS-def and incubated with either FITC-conjugated anti-mouse IgG or tetramethyl rhodamine-conjugated anti-rabbit IgG (Cappel, Durham, NC) at room temperature for 30 min. The cells were subsequently washed and mounted onto slides with Aquamount (Lerner Laboratories, New Haven, CT).

Cell Extractions and Immunoblot Analysis

Transiently transfected cells were washed with PBS-def and subsequently lysed and incubated in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5 mM PMSF, 10 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A in ice for 15 min. The lysate was centrifuged at 4°C at 430,000 g for 1 h. The resulting detergent-soluble fraction was further centrifuged at the same speed for 30 min to ensure complete removal of insoluble materials. The detergent-insoluble pellet was digested with 0.2 mg/ml of DNase I in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM PMSF and 2 μg/ml leupeptin at 4°C for 15 min and was then centrifuged at 430,000 g for 1 h. The pellet was subsequently washed with 10 mM Tris-HCl (pH 7.5), 150 mM NaCl containing the four protease inhibitors as described above, and was centrifuged again at 430,000 g for 1 h. The pellet was boiled in SDS-sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.01% Bromophenol blue, and was then centrifuged in a microfuge to remove insoluble materials, and the resulting supernatant was designated as the Triton X-100-insoluble fraction.

Proportional amounts of the Triton X-100-insoluble and -soluble fractions were electrophoresed in SDS-10% polyacrylamide gels (Laemmli, 1970) and were subsequently electrotransferred to nitrocellulose filters

(Towbin et al., 1979). The blots were incubated in PBS-def containing 5% dry milk at room temperature for 30 min. After several washes with PBS-def, the blots were incubated in PBS-def containing 3% BSA and primary antibodies at 4°C overnight. The blots were then washed and incubated in PBS-def containing 3% BSA, 0.05% Triton X-100, and ^{125}I-labeled protein A or goat anti–mouse Ig at room temperature for 1 h. Subsequently, the blots were washed several times in PBS-def containing 0.05% Triton X-100 and were exposed to XAR-5 film (Eastman-Kodak Co., Rochester, NY) with an intensifying screen at −70°C. The relative amounts of proteins in the detergent-insoluble and -soluble fractions were measured by densitometric scanning of the autoradiograms.

To prepare total cellular proteins, transiently transfected cells were washed with PBS-def and subsequently lysed with 62.5 mM Tris-HCl (pH 6.8) containing 1% SDS. The lysates were boiled for 10 min and centrifuged at 13,000 g for 5 min. Concentrations of the resulting supernatants containing total cellular proteins were determined by Bradford assay (Bradford, 1976). To compare the levels of the accumulated NFTPs between the cultures transfected with single NFTP expression constructs and those cotransfected with two NFTP expression constructs, equal amounts of total cellular proteins from these cultures were boiled in SDS-sample buffer, electrophoresed in SDS-PAGE, and electrotransferred to nitrocellulose filters. The resulting blots were immunostained as described above. Relative amounts of the proteins were measured by densitometric scanning of the autoradiograms.

Results

Coassembly of α-internexin with Vimentin to Form Filament Networks

To determine whether α-internexin can incorporate into preexisting vimentin networks as the NFTPs have been observed to do, mouse Ltk⁻ cells were transfected with expression constructs of α-internexin driven by either the Rous sarcoma virus long terminal repeat (pRSV-α) or the 1.2-kb 5′ flanking region of the α-internexin gene (pαP-α). Immunofluorescence staining of both stable and transient transfectants indicated that α-internexin, a type IV IF, does in fact incorporate into the endogenous type III vimentin inter-

mediate filament network in Ltk⁻ cells (data not shown). Similarly, when an expression construct of rat vimentin, pRSV-Vim, was transiently cotransfected with pRSV-α into the human adrenal carcinoma cell line SW13cl.2Vim⁻, which lacks its own cytoplasmic IF network, the exogenous vimentin coassembled with α-internexin to form filaments (Fig. 1).

Self-Assembly Competency and Coassembly of Type IV IF Proteins

To study the self- and coassembly of α-internexin and the NFTPs, the human adrenal carcinoma cell line SW13cl.2-Vim⁻, which lacks its own cytoplasmic IF network, was employed for all transient transfection experiments described below. Since it is possible that vimentin-expressing revertant cells might arise during culturing, transfected cells were routinely examined for anti-vimentin antibody staining to make certain that assembly had indeed occurred in the absence of a preexisting vimentin network.

Cells transiently transfected with pRSV-α displayed filamentous staining (Fig. 2 A). Similar results were obtained in transfections employing pαP-α (not shown), except that lower levels of α-internexin were expressed under the α-internexin 5′ flanking region, whose promoter strength has previously been shown to be weaker than that of the RSV promoter (Ching and Liem, 1991). Apparently, α-internexin by itself was capable of forming extensive fibrillar networks. In contrast, none of the NFTPs were capable by themselves of forming such networks in cells transfected with pRSVi-NFL1(+), pRSVi-NFM1(+), or pRSVi-NFH1(+); instead, fine, punctate staining patterns were observed (Fig. 2, B–D). Immunoblot analysis of cellular proteins from transiently transfected SW13cl.2Vim⁻ cells showed that NF-L and α-internexin were present only in the Triton X-100-insoluble fraction, whereas NF-M and NF-H were detected in both the
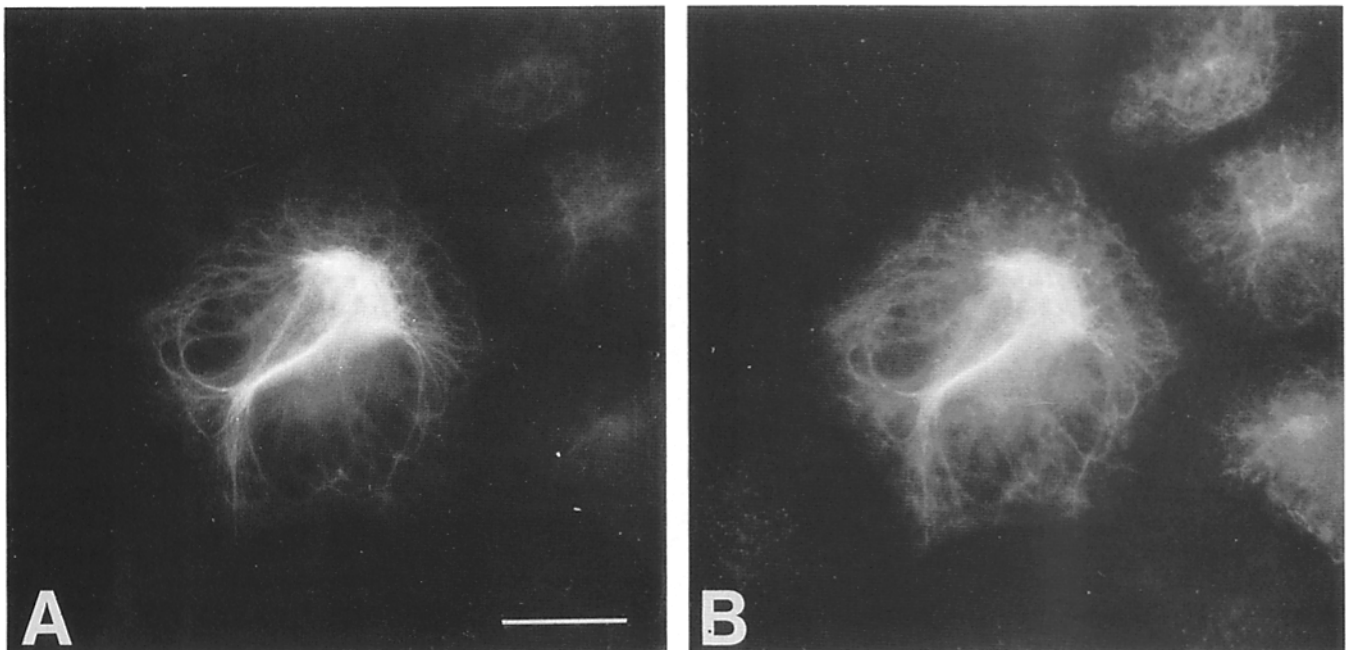
Figure 1. Coassembly of α-internexin with vimentin into filamentous networks. SW13cl.2Vim⁻ cells transiently transfected with pRSV-α and pRSV-Vim were stained with mouse monoclonal anti-α-internexin (*A*) and rabbit polyclonal anti-vimentin (*B*) antibodies. Bar, 20 μm.
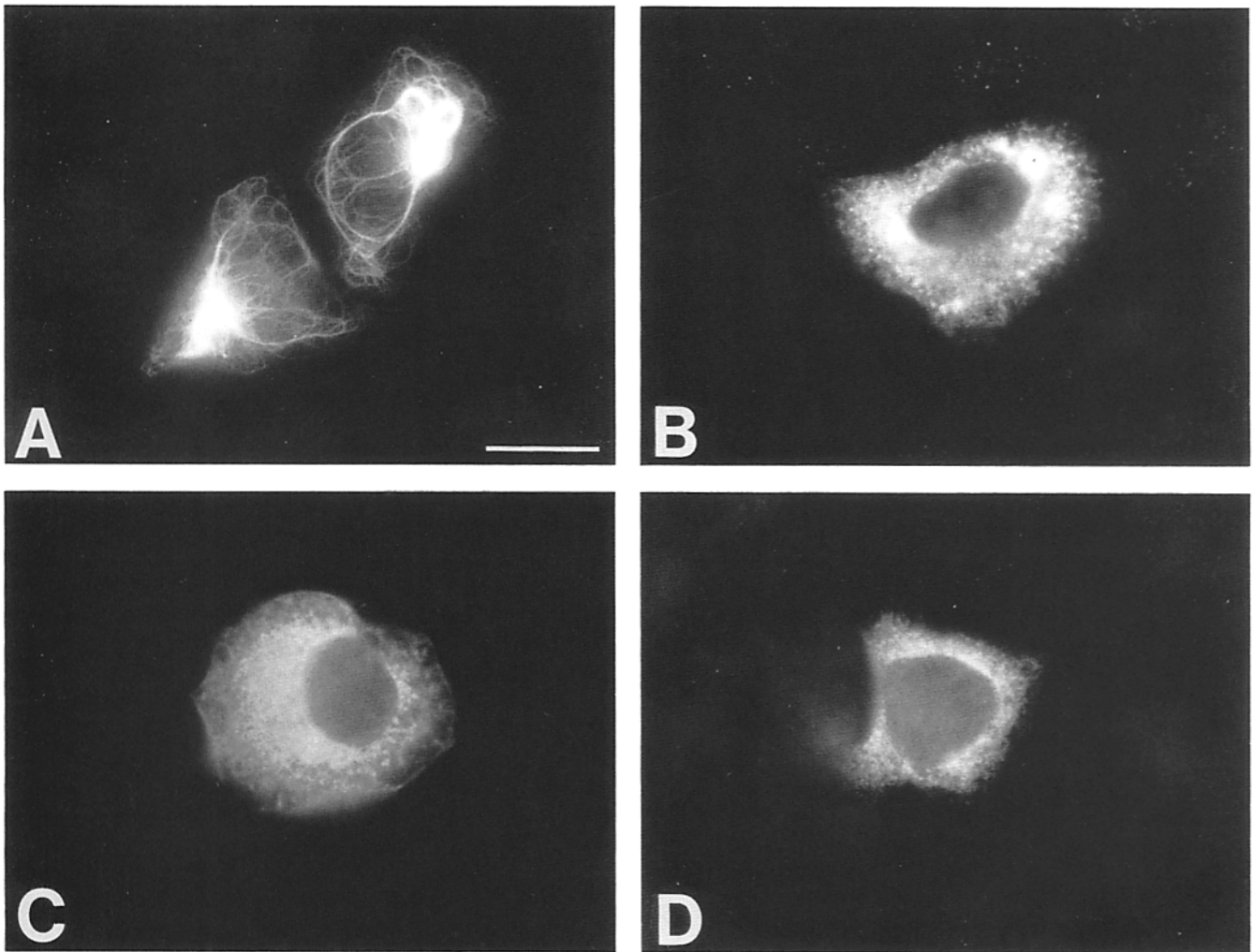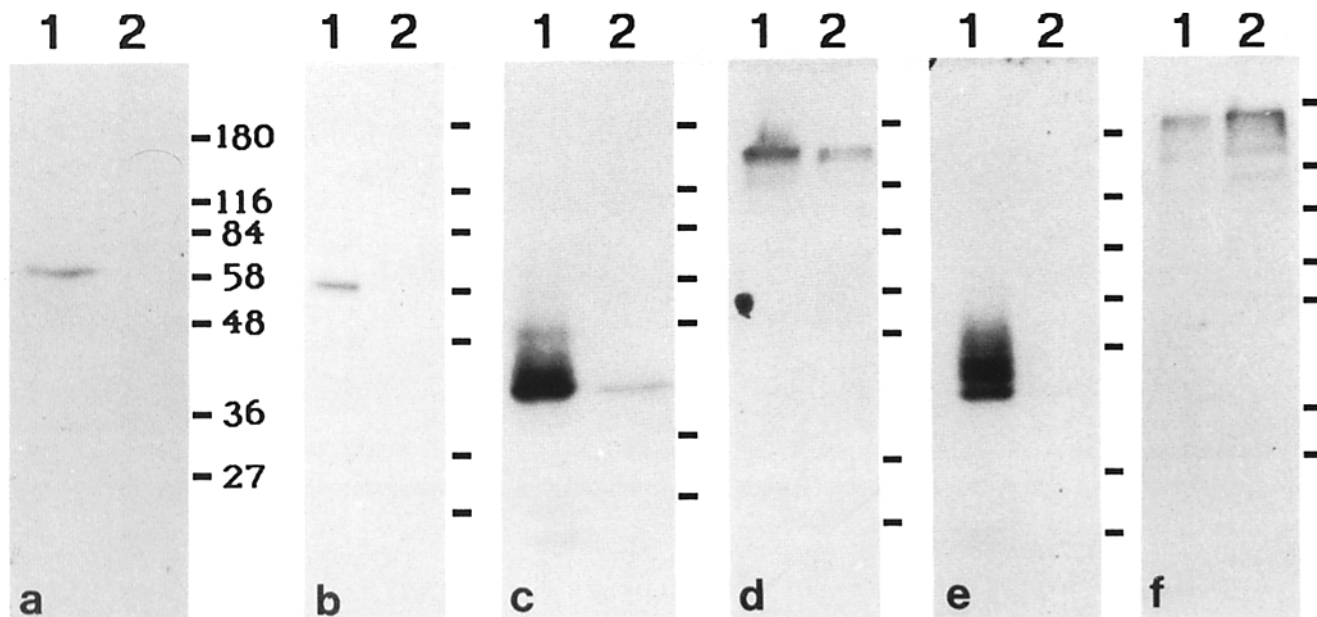
Figure 2. Self-assembly of type IV IF proteins. SW13cl.2Vim⁻ cells transiently transfected singly with pRSV-α (*A*), pRSVi-NFLl(+) (*B*), pRSVi-NFMl(+) (*C*), or pRSVi-NFHl(+) (*D*) were stained with mouse monoclonal anti-α-internexin, rabbit polyclonal anti-NF-L, rabbit polyclonal anti-NF-M, and mouse monoclonal anti-NF-H antibodies, respectively. Bar, 20 μm.

Triton X-100-insoluble and -soluble fractions (Fig. 3, *A, B, D,* and *F,* and Table I). The presence of the NFTPs in the insoluble fractions suggest that the transfected proteins were probably present in some kind of polymeric form of a higher order than the tetrameric stage, despite their inability to form filamentous structures. It should be noted that α-internexin as well as NF-L were distributed equally between the Triton X-100-insoluble and -soluble fractions when the cell extracts were centrifuged at 13,000 *g* for 30 min (data not shown) instead of at 430,000 *g* as described above. Apparently, a centrifugal force higher than 13,000 *g* was required to spin down the insoluble filaments or polymeric structures from the transfected cells, which represent a very small population among the much more numerous IF-negative, untransfected SW13cl.2Vim⁻ cells.

To examine whether α-internexin is able to coassemble with any one of the NFTPs into filamentous networks, pRSV-α was transiently cotransfected with pRSVi-NFLl(+), pRSVi-NFMl(+), or pRSVi-NFHl(+) into SW13cl.2Vim⁻ cells. As shown in Fig. 4, *A-F,* the filamentous staining pat-

terns were similar to those observed in cells transfected with pRSV-α alone, and individual anti-NFTP antibody staining coincided with the anti-α-internexin antibody staining. These results indicated that α-internexin was capable of interacting with each of the NFTPs to form long fibrillar structures, which at the light microscopic level were qualitatively indistinguishable from those composed of only α-internexin. It should be noted that the morphologies of the filament networks resulting from these cotransfections were somewhat heterogeneous; the variety of filamentous patterns shown in Fig. 4, *A-F* were seen in all of these cotransfections. Interestingly, cotransfection of pαP-α with pRSVi-NFMl(+) resulted in formation of filamentous fragments in some (∼20%) of the transfected cells, although long, filamentous networks were observed in most of the transfected cells (Fig. 4, *G* and *H*). These filamentous fragments were absent in cells transfected with pRSV-α and pRSVi-NFMl(+).

To determine whether NF-L and NF-M can coassemble into filamentous structures, SW13cl.2Vim⁻ cells were transiently transfected with pRSVi-NFLl(+) and pRSVi-

Figure 3. Western blot analysis of type IV IF proteins from extracts of transiently transfected cells. Cell extracts of transfected SW13cl.2Vim⁻ cells were separated into Triton X-100-insoluble (lane *1*) and -soluble fractions (lane *2*) by centrifugation at 430,000 *g*. Proteins were electrophoresed in SDS-10% polyacrylamide gels and were subsequently transferred to nitrocellulose filters. The samples in the blots were from cells transfected singly with pRSV-α (*a*); pRSVi-NFLl(+) (*b*); L1-402 (*c*); pRSVi-NFM1(+) (*d*); M1-421 (*e*); and pRSVi-NFH1(+) (*f*). The blots were immunostained with mouse monoclonal anti-α-internexin (*a*), rabbit polyclonal anti-NF-L (*b*), AbNFL_n (*c*), rabbit polyclonal anti-NF-M (*d*), AbNFM_n (*e*), and mouse monoclonal anti-NF-H (*f*) antibodies. Bars indicate the position of the prestained protein markers with molecular masses of 180, 116, 84, 58, 48, 36, and 27 kD.

NFM1(+). Immunofluorescence staining of the transfected cells showed that NF-L and NF-M interacted to form filaments (Fig. 5, *A* and *B*). Similar filamentous staining was observed in cells transfected with pRSVi-NFL1(+) and pRSVi-NFH1(+), indicating that NF-L also coassembled with NF-H into filamentous structures (Fig. 5, *C* and *D*). In both cotransfections, filamentous fragments similar to those seen in cotransfection of pαP-α with pRSVi-NFM1(+) were observed in 15–20% of the transfected cells (data not shown).

In contrast, cotransfection of pRSVi-NFM1(+) with pRSVi-NFH1(+) did not result in filamentous staining (Fig. 5, *E* and *F*). Instead, NF-M and NF-H appeared to be colocalized in a fine, punctate staining pattern similar to that observed in cells singly transfected with pRSVi-NFM1(+) or pRSVi-NFH1(+).

Table I. Relative Amounts of Neuronal IF Proteins in the Triton X-100-Insoluble and -Soluble Fractions from Transiently Transfected SW13cl.2Vim⁻ Cells

| Protein	% Insoluble	% Soluble
α-internexin	100	0
NF-L	100	0
L1-402	97	3
NF-M	80	20
M1-421	100	0
NF-H	39	61

The relative amounts of the neuronal IF proteins present in the detergent-insoluble and -soluble fractions were determined by densitometric scanning of the autoradiograms of the Western blots shown in Fig. 3. Autoradiograms of several different exposures were used for scanning in order to assure accurate measurements.

It should be noted that 20 μg of single DNA constructs were used for the transfections of pRSVi-NFL1(+), pRSVi-NFM1(+), or pRSVi-NFH1(+) shown in Fig. 2, whereas 10 μg each of the two DNA constructs were used for the cotransfections shown in Fig. 5 (see Materials and Methods). Even when the amounts of DNA used for transfections were increased to 30 μg, cells transiently transfected with a single NFTP expression construct all yielded fine, punctate staining patterns such as those shown in Fig. 2. Table II shows that the levels of the accumulated NFTPs in cells transfected with two DNA constructs vary from 70 to 90% of the levels of the proteins in the cells transfected with a single DNA construct. Similar variations were seen in duplicates of these experiments. The slightly higher levels of protein in the cells transfected with the single constructs are probably due to the higher amounts of DNA (20 μg) used for these transfections. These data are directly relevant to the immunofluorescence results shown in Figs. 2 and 5, since the same amounts of DNA were used for each experiment. Within the limits of the transient transfection techniques, these results suggest that the lack of filament formation observed in cells transfected with pRSVi-NFL1(+), pRSVi-NFM1(+), or pRSVi-NFH1(+) alone is unlikely to be due to insufficient levels of accumulated NFTPs, but is rather due to some intrinsic inability of these proteins to form homopolymeric IF structures.

Effects of Carboxyl-Terminal Deletions in NF-L and NF-M on Their Coassembly with Other Type IV IF Proteins

When L1-402, encoding a truncated NF-L protein in which
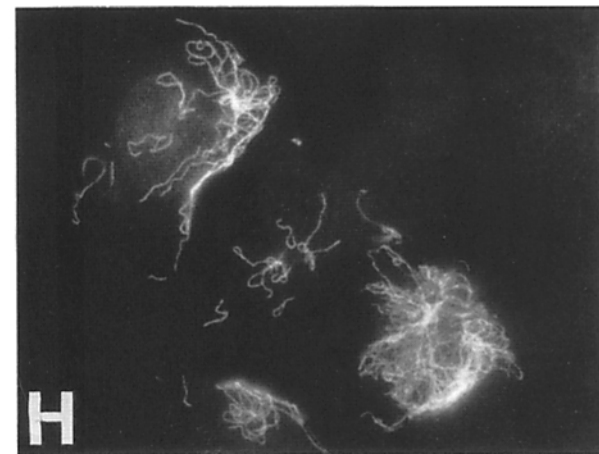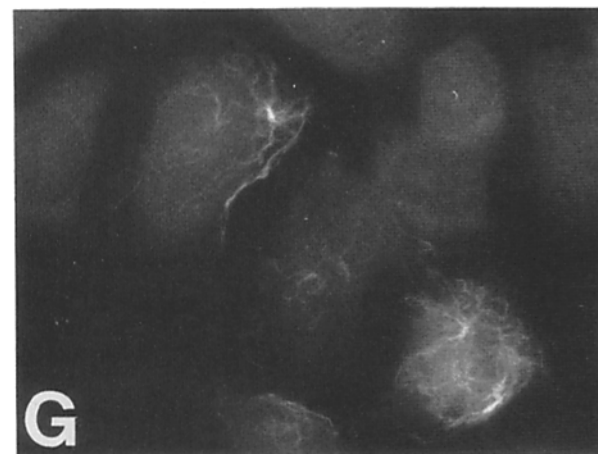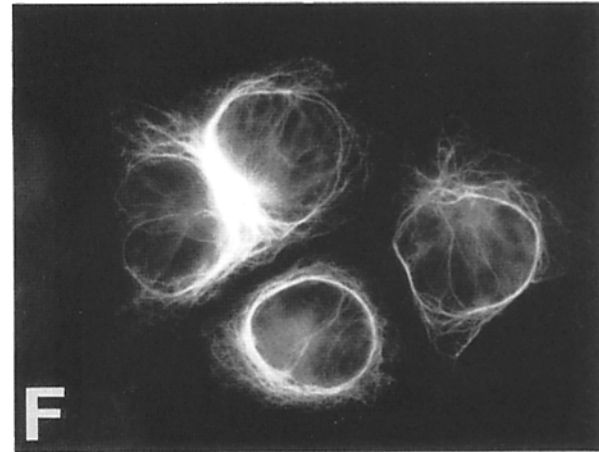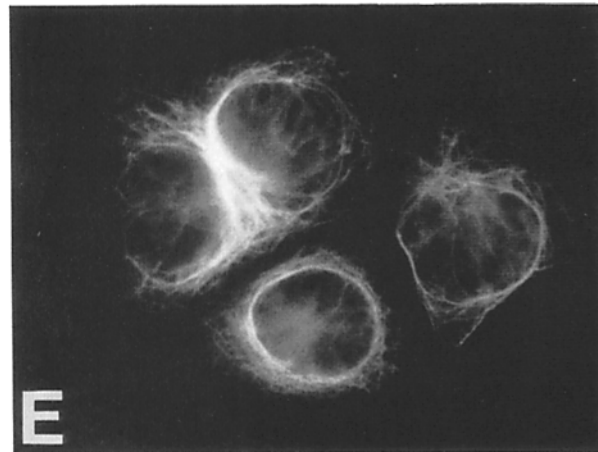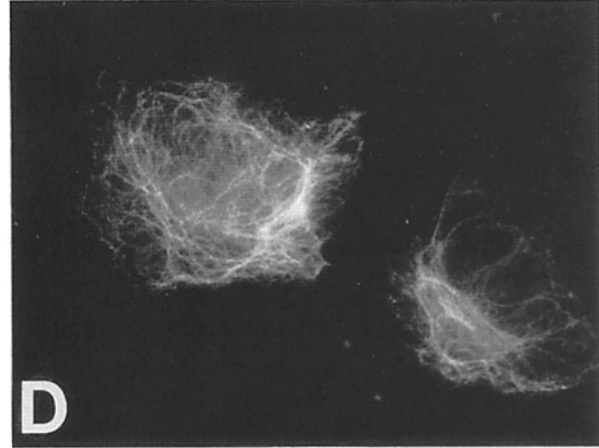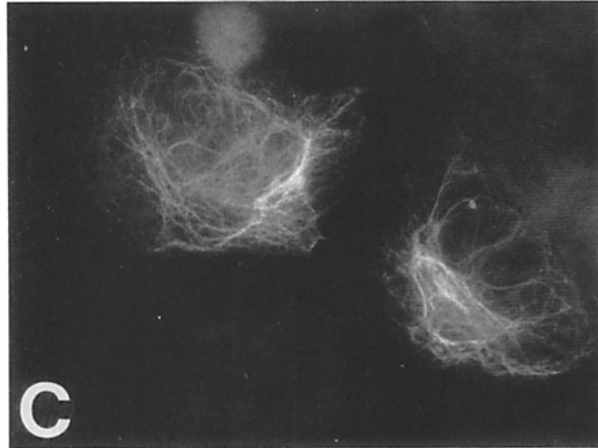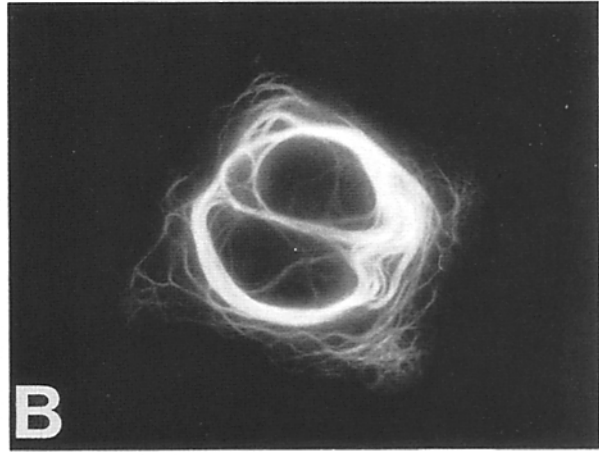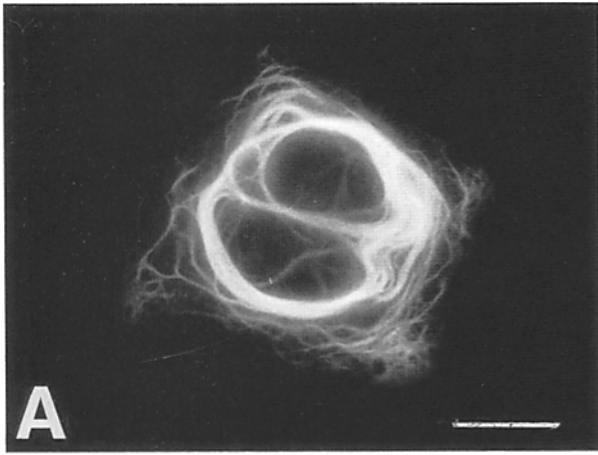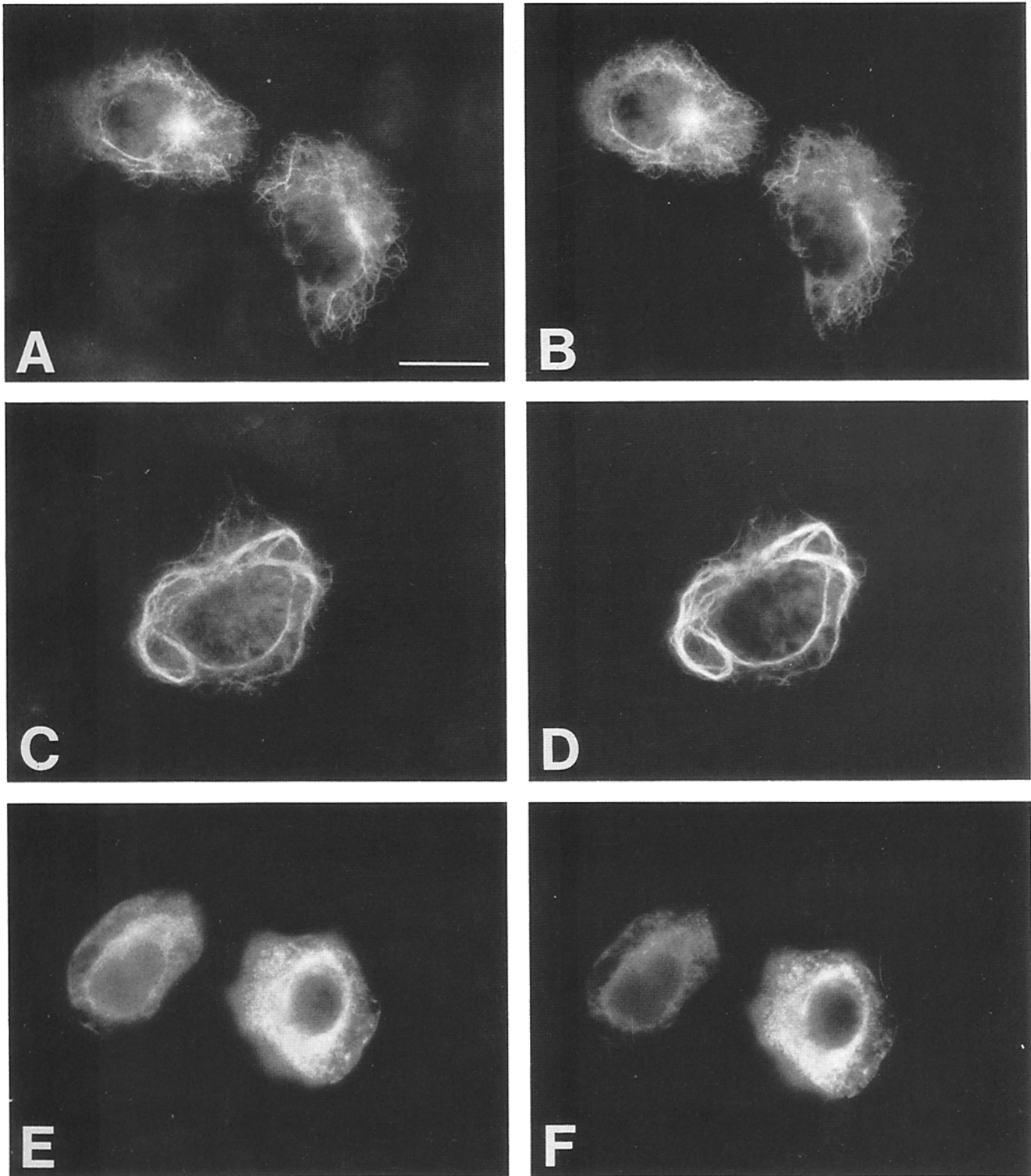
Figure 5. Coassembly of neurofilament triplet proteins. SW13cl.2Vim⁻ cells from transient cotransfection of pRSVi-NFL1(+) with pRSVi-NFM1(+) (*A* and *B*), pRSVi-NFL1(+) with pRSVi-NFH1(+) (*C* and *D*), and pRSVi-NFM1(+) with pRSVi-NFH1(+) (*E* and *F*) were stained with mouse monoclonal anti-NF-L (*A*), rabbit polyclonal anti-NFM (*B* and *E*), rabbit polyclonal anti-NF-L (*C*), and mouse monoclonal anti-NF-H antibodies (*D* and *F*). Bar, 20 μm.

Figure 4. Coassembly of α-internexin with neurofilament triplet proteins. SW13cl.2Vim⁻ cells from transient cotransfection of pRSV-α with pRSVi-NFL1(+) (*A* and *B*), pRSVi-NFM1(+) (*C* and *D*), or pRSVi-NFH1(+) (*E* and *F*), and of pαP-α with pRSVi-NFM1(+) (*G* and *H*) were stained with mouse monoclonal antibodies to α-internexin (*A*, *C*, and *G*) or NF-H (*F*) and rabbit polyclonal antibodies to NF-L (*B*), NF-M (*D* and *H*), or α-internexin (*E*). Bar, 20 μm.

Table II. Comparison of the Levels of the Accumulated NFTPs between SW13cl.2Vim⁻ Cells Transiently Transfected with Single NFTP Expression Constructs and Those Transiently Cotransfected with Two NFTP Expression Constructs

	NF-L + NF-M	NF-L + NF-H
NF-L	0.74	nd
NF-M	0.76	—
NF-H	—	0.92

Transfections were performed as described for those shown in Fig. 2 and Fig. 5. The relative amounts of the neuronal IF proteins present in the transfected cells were determined by densitometric scanning of the autoradiograms of Western blots of total cellular proteins. Values for NF-L, NF-M, and NF-H in the cotransfections are expressed as ratios of the amounts observed in single-construct transfections.

all but two amino acid residues of the tail domain is removed (Chin et al., 1991; see also Fig. 6 A), was transfected into SW13cl.2Vim⁻ cells, tubular-vesicular structures were observed which were stained by the anti-NFL$_n$ antibody (Fig. 7, A and B). These structures appeared to be located within the cytoplasm and often clustered around one side of the nucleus. Immunoblot analysis showed that the truncated protein expressed in the transfected cells was present mainly in the Triton X-100-insoluble fraction (Fig. 3 C). On the other hand, cotransfection of L1-402 with either α-internexin,

pRSVi-NFM1(+), or pRSVi-NFH1(+) yielded filamentous staining patterns reminiscent of those observed with intact NF-L coassembled with α-internexin, NF-M or NF-H (Fig. 7, C-H). However, in ∼20–30% of the cells cotransfected with pRSV-α, tubular-vesicular structures similar to those comprised of only the L1-402 mutant protein were observed, which were stained by both the anti-NFL$_n$ and anti-α-internexin antibodies. These tubular-vesicular structures were also found in 10% of the cells cotransfected with pRSVi-NFM1(+) and 20% of those cotransfected with pRSVi-NFH1(+). Formation of these tubular-vesicular structures is probably due to the overexpression of the L1-402 mutant protein in these cells, causing the collapse of any filaments formed and preventing α-internexin from associating with itself to form homopolymeric IF structures. Alternately, it may be due to partial degradation of the L1-402 protein resulting in deletion into coil 2 of the rod domain, the type of deletion which has been shown to collapse the endogenous vimentin network and which yields similar kinds of tubular-vesicular structures in transfected Ltk⁻ cells (Chin et al., 1991). These two possibilities are both likely, and not mutually, exclusive. Nevertheless, the results presented here show that the carboxyl-terminal tail domain is dispensable for normal coassembly of NF-L with α-internexin, NF-M, or NF-H into filaments.

M1-421 encodes a tailless NF-M which has all but 10 residues removed from its tail domain (Fig. 6 B). Transfec-
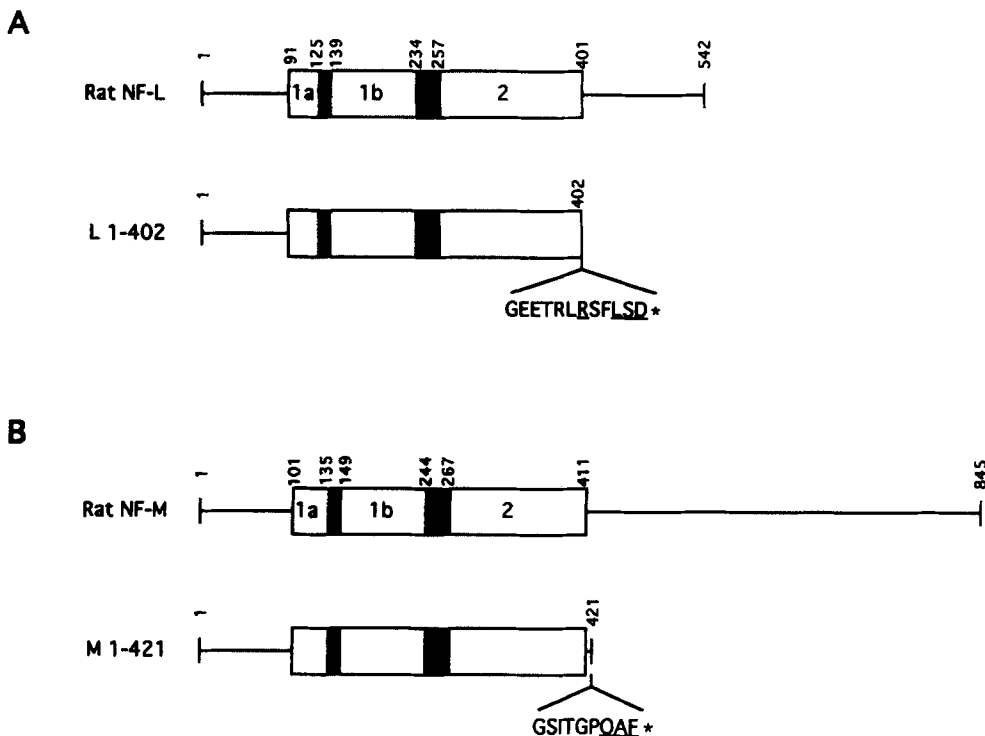
*Figure 6.* Schematic illustrations of the truncated NF-L and NF-M proteins. NF-L (A) and NF-M (B) and their carboxy-terminal deletion mutants are shown. The helical domains are represented by open rectangles and the linker regions by closed rectangles. Amino acid positions of the domain junctions are indicated with numbers. Amino acid sequences at the deletion junctions of the mutants are shown with one-letter codes, and additional amino acid residues generated from clonings of the DNA constructs are underlined. Asterisks indicate the position of the stop codon. It should be noted that the amino acid sequence at the deletion junction of L1-402 reported by Chin et al. (1991) is incorrect; the correct sequence is shown here. Also, the Phe (F) residue is at position 403; the tail domain of L1-402 thus retains two amino acid residues.

Figure 7. Assembly characteristics of the carboxyl-terminal deletion mutant of NF-L. SW13cl.2Vim⁻ cells from transient transfections of L1-402 (A and B), L1-402 and pRSV-α (C and D), L1-402 and pRSVi-NFM1(+) (E and F), and L1-402 and pRSVi-NFH1(+) (G and H) were stained with rabbit polyclonal antibody AbNFL$_n$ (A-C, E and G) and mouse monoclonal antibodies to α-internexin (D), NF-M (F), and NF-H (H). Bar, 20 μm.

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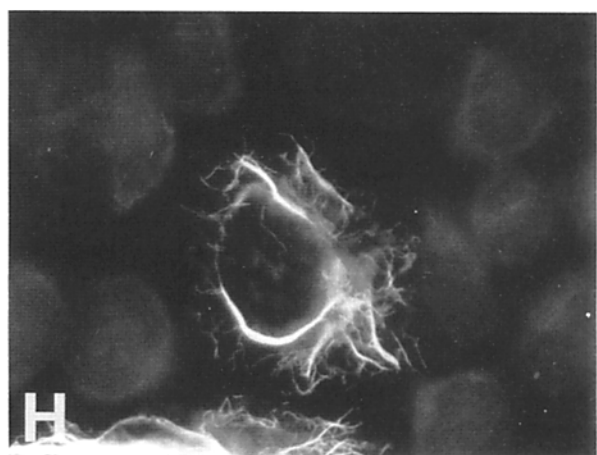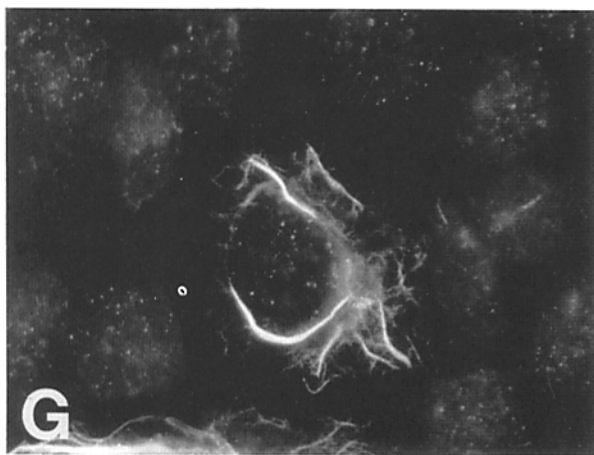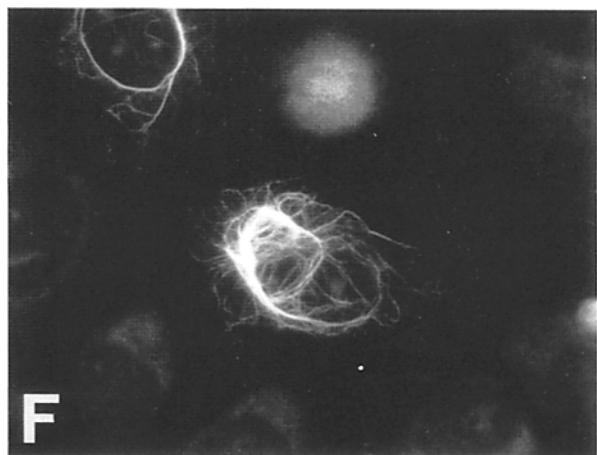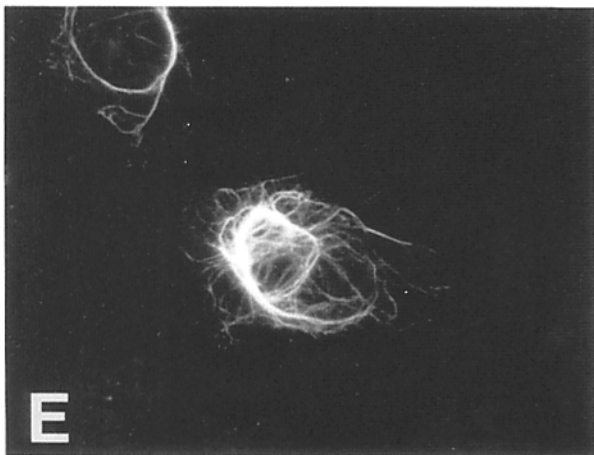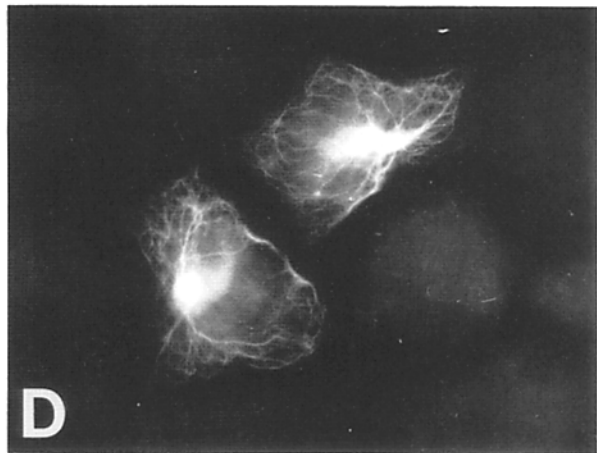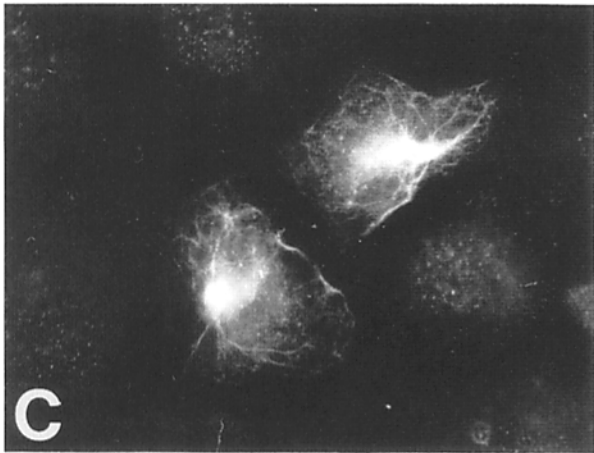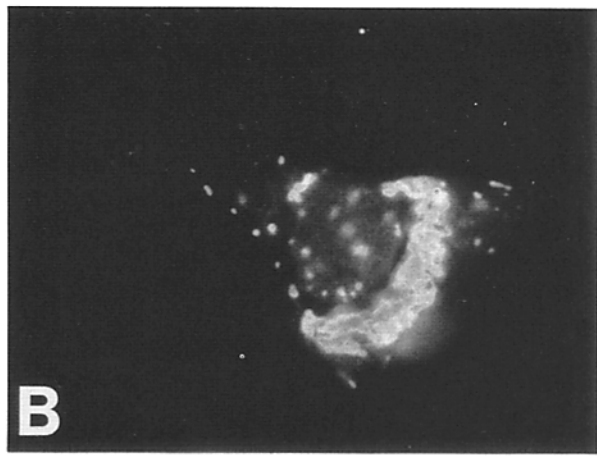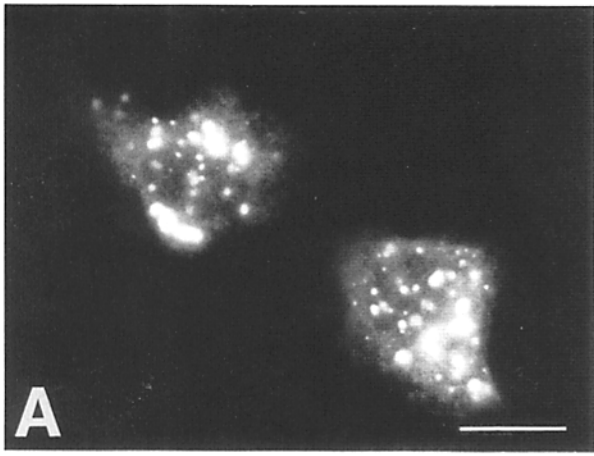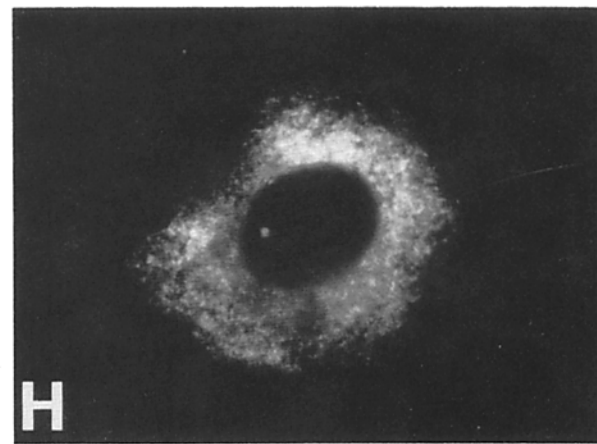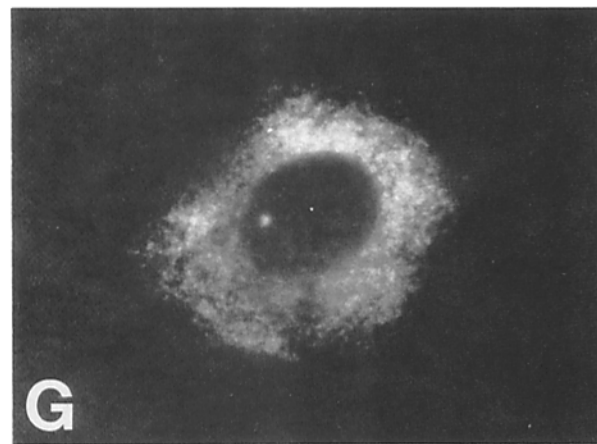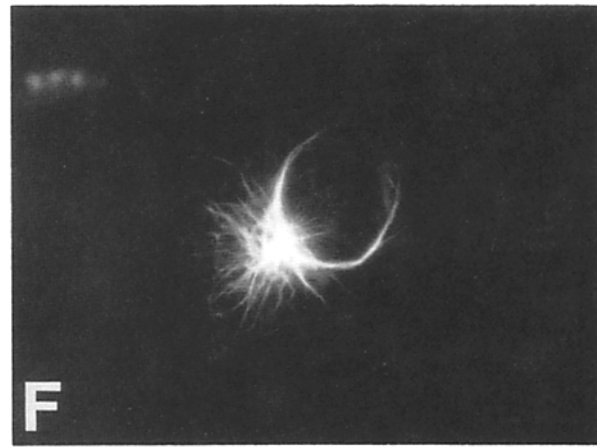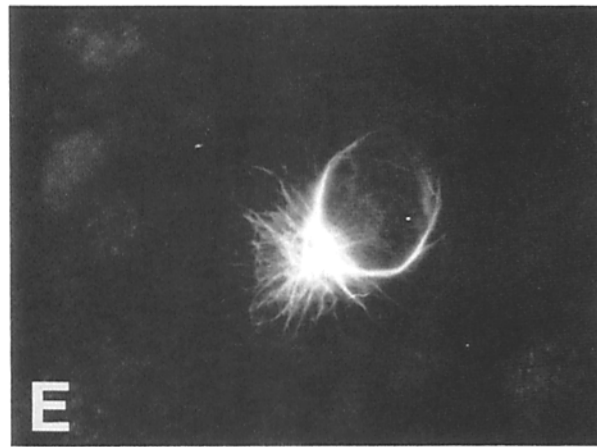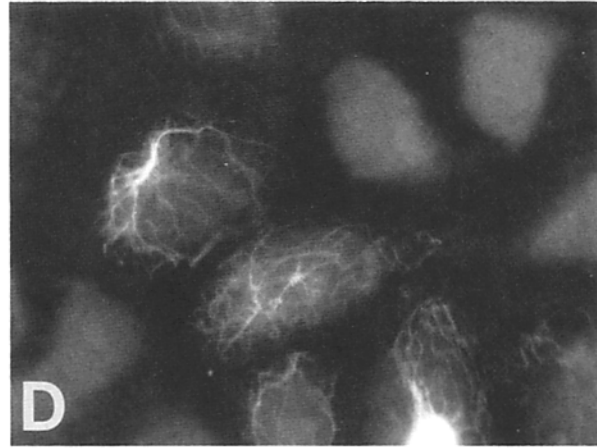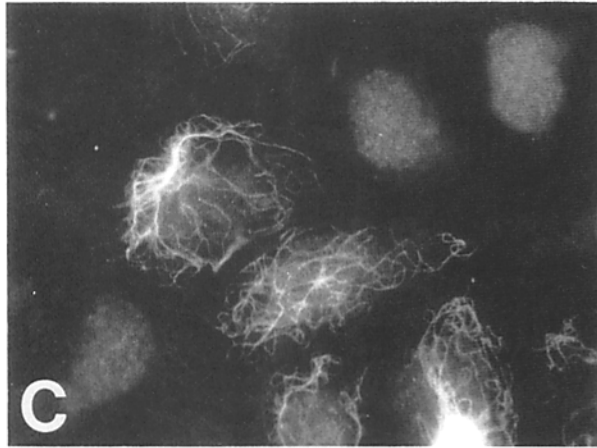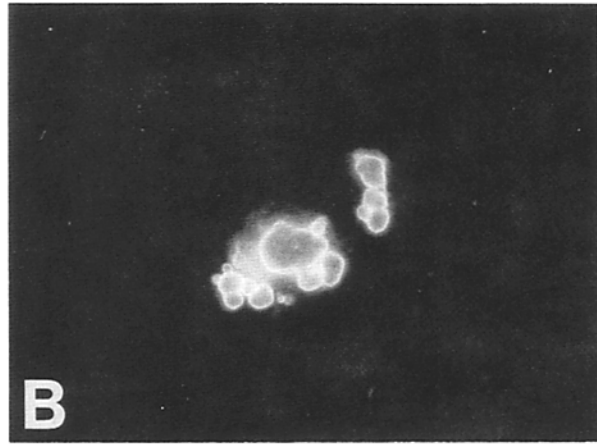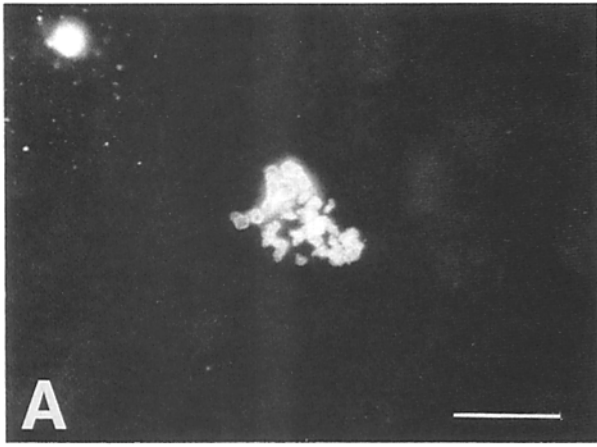
tion of this tailless NF-M construct into SW13cl.2Vim⁻ cells resulted in the formation of tubular-vesicular structures in the cytoplasm (Fig. 8, *A* and *B*), which often clustered asymmetrically around one side of the nucleus. The mutant protein was present in the Triton X-100-insoluble fraction of the transfected cells, and a substantial proportion of the protein appeared to be partially degraded from the carboxyl end (Fig. 3 *E* and Table I). Cotransfection of M1-421 with pRSV-α resulted in formation of filaments stained by both the anti-NFMₙ and anti-α-internexin antibodies (Fig. 8, *C* and *D*). Cotransfection of M1-421 with pRSVi-NFL1(+) also yielded a filamentous staining pattern (Fig. 8, *E* and *F*). When M1-421 was cotransfected with pRSVi-NFH1(+), punctate staining patterns similar to those observed with cotransfection of pRSVi-NFM1(+) and pRSVi-NFH1(+) were seen (Fig. 8, *G* and *H*). However, in ~10% of the cells cotransfected with pRSV-α and 30% of the cells cotransfected with either pRSVi-NFL1(+) or pRSVi-NFH1(+), tubular-vesicular structures were observed. The formation of these tubular-vesicular structures is probably due to overexpression and/or partial degradation of a proportion of the M1-421 mutant protein, as is the case for the L1-402 mutant. Overall, these results suggest that the tail region is dispensable for normal coassembly of NF-M with α-internexin or NF-L into filaments, and with NF-H into heteropolymers.

Discussion

Our present studies have yielded several interesting results regarding the assembly characteristics of the type IV neuronal IF proteins. Like the NFTPs, α-internexin was capable of coassembly with vimentin to form a filamentous network in transfected cells. But unlike any of the NFTPs which by themselves were incapable of homopolymeric filament formation, α-internexin formed long fibrillar structures by itself in transfected SW13cl.2Vim⁻ cells. This capacity for self-assembly into long filaments in the absence of any preexisting IF network is interesting in light of the finding that α-internexin is the only neuronal IF present in many young postmigratory neurons of the developing rat CNS at a time when the NFTPs are not yet expressed, and also in the parallel fibers of the cerebellar granular cells in the adult, which appear to lack other IF proteins (Kaplan et al., 1990; Fliegner, K. H., M. P. Kaplan, J. E. Pintar, T. L. Wood, and R. K. H. Liem, manuscript submitted for publication). Moreover, α-internexin could coassemble with each of the NFTPs to form filamentous networks in the transfected SW13cl.2Vim⁻ cells, an observation consistent with previous immunofluorescence studies which showed the colocalization of these proteins in axons. Taken together, these data suggest that in addition to a possible structural role in stabilizing small-diameter axons (such as parallel fibers or growing processes), α-internexin may play an additional role in the normal incorporation of the other NFTPs into filamentous networks.

Although each of the NFTPs by itself was unable to form filamentous networks in transiently transfected SW13cl.2-Vim⁻ cells, the presence of these proteins in the Triton X-100-insoluble fractions of the cell extracts indicates that they are probably capable of forming homopolymers of a higher order than the tetrameric stage. NF-M and NF-H, but not NF-L, were also detected in the Triton-soluble fractions suggesting that the NFTPs may differ in their competency to form these higher order structures. Such differences may account for the ability of NF-L to polymerize readily into homopolymeric IF structures under certain in vitro conditions, and the inability of NF-M and NF-H to do so under the same conditions (Geisler and Weber, 1981; Liem and Hutchison, 1982; Hisanaga and Hirokawa, 1990). When extractions of the transfected cells were performed in the same buffer system without addition of Triton X-100, NF-M and NF-H were found in the supernatant, indicating that they also may exist in the cytosolic, soluble form (data not shown). Further extraction of the remaining pellets with Triton X-100 showed that these two proteins were also present in the resulting detergent-soluble fractions. Taken together, these results suggest that NF-M and NF-H are present in the cytoplasm of the transfected cells in three distinct forms: insoluble (Triton X-100-insoluble), membrane-associated (Triton X-100-soluble), and soluble. Soellner et al. (1985) have reported that in cultured cells a small pool of vimentin exists in a cytosolic, soluble tetrameric form. It is thus possible that the membrane-associated and soluble forms of NF-M and NF-H in these transfected cells are tetramers and/or dimers.

NF-L was able to interact with NF-M or NF-H to form IF structures in the transfected cells, whereas NF-M and NF-H were unable to coassemble into filaments. While these results are consistent with cell-free in vitro polymerization studies which showed that NF-M and NF-H require NF-L to copolymerize into filaments (Geisler and Weber, 1981; Liem and Hutchison, 1982), the competency of NF-L to form homopolymeric IF structures in these in vitro studies was not observed in the transfected cells. Since cell-free in vitro polymerization depends on myriad experimental parameters such as pH, ionic strength, and Mg^{2+} concentration, it is not entirely surprising to see that NF-L exhibited somewhat different assembly properties in these studies. However, the present observations that NF-L could coassemble with NF-M or NF-H to form filaments in the absence of any preexisting IF network are in agreement with the fact that mammalian neurofilaments in vivo are formed from heteropolymeric IF structures composed of all three NFTPs (Willard and Simon, 1981; Hirokawa et al., 1984). Moreover, they suggest that the assembly process may involve NF-L/NF-M and NF-L/NF-H complexes acting as the intermediate building blocks for neuronal IFs. As previously shown for the assembly of cytokeratin, an obligate heteropolymer, a molecular recognition event appears to be involved in the nucleation step of the neuronal IF assembly process, since IF formation requires specific NF-L/NF-M or NF-L/NF-H heteropolymerizations. In contrast to the kera-

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Figure 8. Assembly characteristics of the carboxyl-terminal deletion mutant of NF-M. SW13cl.2Vim⁻ cells from transient transfections of M1-421 (*A* and *B*), M1-421 and pRSV-α (*C* and *D*), M1-421 and pRSVi-NFL1(+) (*E* and *F*), and M1-421 and pRSVi-NFH1(+) (*G* and *H*) were stained with rabbit polyclonal antibody AbNFMₙ (*A–C, E* and *G*) and mouse monoclonal antibodies to α-internexin (*D*), NF-L (*F*), and NF-H (*H*). Bar, 20 μm.

tins, however, the NFTPs are able to form stable homopolymers of higher order than the tetrameric stage when present alone in transfected cells, as suggested by the presence of these proteins in the Triton X-100-insoluble fraction. Heteropolymerization is apparently more favorable than homopolymerization, as indicated by the prevalence of heterofilaments, and the absence of observable homopolymeric structures, in transfected cells expressing two different NFTPs (e.g., NF-L and NF-M or NF-H).

It is interesting to note that filamentous fragments were observed in a minor population of cells transfected with pRSVi-NFM1(+) and pαP-α (Fig. 4, G and H). Similar results were obtained from cotransfection of pRSVi-NFL1(+) with pRSVi-NFM1(+) or pRSVi-NFH1(+). It seems that once expressed, even at low levels insufficient for formation of extensive filamentous networks, these coexpressed neuronal IF proteins coassembled and elongated into filamentous fragments. However, since transfection efficiencies and levels of exogenous gene expression vary in transiently transfected cells, the stoichiometric amounts of these proteins required for filament formation could not be determined.

The time course of developmental expression of the type IV proteins and the inability of any of the NFTPs to form homopolymeric filamentous networks suggest that α-internexin may act as a scaffold for the formation of neuronal IFs during early development. Expression of α-internexin appears to precede that of the NFTPs throughout most of the developing nervous system (Kaplan et al., 1990; Fliegner, K. H., M. P. Kaplan, J. E. Pintar, T. L. Wood, and R. K. H. Liem, manuscript submitted for publication). As the other NFTPs are expressed, they may incorporate directly into the preexisting α-internexin network, and/or coassemble with α-internexin into IF networks. As development continues, expression of α-internexin decreases and that of the NFTPs increases, so that α-internexin in the neuronal IF network is progressively replaced with the NFTPs. In this way, α-internexin and the NFTPs could each accomplish their possible roles of stabilizing small and/or growing axons and regulating the caliber of large axons, respectively, in development and in the adult.

Transient transfections in SW13cl.2Vim⁻ cells with the carboxyl-terminal deletion constructs L1-402 and M1-421 demonstrated that the entire tail regions of NF-L and NF-M are dispensable for normal coassembly of each of these two proteins with the other type IV IF proteins to form filaments. However, these deletions appeared to affect the homopolymerization of both mutants, resulting in the formation of Triton X-100-insoluble tubular-vesicular structures in the cytoplasm of cells transfected with the L1-402 or M1-421 constructs alone. In contrast to the wild-type NF-M, the M1-421 mutant was present only in the Triton X-100-insoluble fraction of the transfected cell extracts (Table I). The observed tubular-vesicular structures appeared at the level of light microscopy to be similar to those seen in the transfected Ltk⁻ cells with truncated NF-L mutants containing carboxyl-terminal deletions into the rod domain, which were shown by electron microscopy to be associated with vesicles in the cytoplasm (Chin et al., 1991). It is thus possible that the tubular-vesicular structures formed by the L1-402 and M1-421 mutants in the SW13cl.2Vim⁻ cells may be similarly associated with cytoplasmic vesicles. It is noteworthy that in the majority of the transfected SW13cl.2Vim⁻ cells, coassembly of the L1-402 or M1-421 mutants with other exogenous type IV IF proteins into filaments prevented the mutants from forming tubular-vesicular structures. Like the wild-type NF-M, the M1-421 mutant did not form filaments with NF-H, but their coassembly resulted in a punctate staining pattern instead of the formation of tubular-vesicular structures. Rather than self-associate, the L1-402 and M1-421 mutants appear to interact and associate preferentially with the other type IV IF proteins and are thus no longer free to form homopolymeric tubular-vesicular structures.

Our present observations with L1-402 and M1-421 in transfected SW13cl.2Vim⁻ cells are consistent with the reports of Chin et al. (1991) and Wong and Cleveland (1990) which showed, respectively, that the L1-402 mutant and a carboxyl-terminal deletion mutant of NF-M retaining only 9 amino acid residues of the tail could incorporate normally into the endogenous vimentin network although they also formed cytoplasmic aggregates in minor populations of the transfected fibroblasts. Since the present study showed coassembly of the tailless NF-L or NF-M mutants with other type IV IF proteins in vimentin-free cells, it eliminates the possibility that the presence of the preexisting vimentin network could mask the effect of the tailless mutants on filament formation. Our results are also similar to those obtained in transfection studies using tailless keratins. While Lu and Lane (1990) reported that a tailless keratin mutant could coassemble into normal filaments only if the other keratin partner within a heterotypic pair was intact, Bader et al. (1991) demonstrated that removal of the tail domain from both types of keratins could still lead to formation of extensive arrays of IFs in transfected mouse 3T3 cells. However, the tail domain appears to play an important role in filament assembly of other IF proteins. A tailless desmin was shown to be incapable of homopolymeric filament assembly in transfected cells lacking preexisting vimentin and desmin networks (Raats et al., 1991). Similarly, a tailless vimentin, which integrated normally into the endogenous vimentin network in transfected vimentin-containing cells, was shown to form short fibrillar structures as well as spheroidal aggregates in transfected vimentin-free cells (Eckelt et al., 1992). It is noteworthy that desmin and vimentin are both type III IF proteins and are able to self-assemble into filamentous structures. In light of these findings, it will be of great interest to characterize the assembly of a tailless α-internexin to determine the role of the tail domain in its homopolymeric filament assembly as well as in coassembly with other type IV IF proteins. It will also be important to investigate whether removal of the tail domain from both NF-L and NF-M can result in their coassembly into filamentous networks.

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