Characterization of Interactions between the Neurofilament Triplet Proteins by the Yeast Two-hybrid System*

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In the adult axon, the neurofilaments (NFs) are heteropolymers formed from the low (NFL), middle (NFM), and high (NFH) molecular weight neurofilament triplet proteins (NFTPs). All three proteins have the basic intermediate filament protein tripartite structure, which consists of a short amino-terminal head region, an α-helical rod region of ∼310 amino acids, and a carboxyl-terminal tail region of variable length. In vitro polymerization studies have shown that only NFL can assemble into homopolymeric 10-nm filaments. The assembly of intermediate filaments, including the NFs, begins with the formation of a coiled-coil dimer involving the α-helical rod domains of two molecules. In order to determine whether homodimers or heterodimers of NFTPs are the preferred intermediates in the assembly of NFs, we have used the yeast two-hybrid system to study the interactions between the different NFTPs. By monitoring the activity of the lacZ reporter gene product, we are able to show that the interactions of NFL with NFM, NFM, or NFH are stronger than the interactions of NFM with NFM or NFH and the interaction of NFH with NFH. These results imply that NFM and NFH are more likely to form heterodimers with NFL than homodimers and are consistent with the inability of NFM and NFH to self-polymerize in vitro and in vivo.

Neurofilaments (NFs)† are a class of intermediate filaments (IFs) that are exclusively expressed in mature neurons in both the central and peripheral nervous systems. They have been suggested to play an important role in the modulation of axonal caliber (1, 2) and are composed of the neurofilament triplet proteins (NFTPs) named NFH, NFM, and NFL for the high, middle, and low molecular weight neurofilament subunits, respectively. Similar to other IF proteins, the NFTPs conform to a tripartite structure consisting of a conserved central α-helical "rod" domain of ∼310 amino acids and variable amino- and carboxyl-terminal head and tail domains (3). The α-helical rod domains of the NFTPs contain long stretches of heptad repeats that mediate the formation of coiled-coil dimers, the common first step in intermediate filament assembly.

The assembly of neurofilaments has been extensively studied both in vitro and in vivo. In vitro, purified NFL can self-assemble into filaments of normal morphology, whereas NFM and NFH only form irregular filamentous aggregates. In the presence of NFL, these two proteins co-assemble into heteropolymeric filaments (4, 5). In transfected cells, each of the NFTPs is able to co-assemble with the endogenous vimentin network (6, 7). In contrast to the in vitro polymerization studies, transfection studies have shown that in the absence of an endogenous vimentin network, none of the NFTPs is able to self-polymerize into a filamentous network. NFL co-assembled with either NFM or NFH into filamentous arrays in the transfected cells, but NFM and NFH could not co-assemble into filaments (8–10).

Like other intermediate filament systems, neurofilaments are thought to assemble through three intermediates, dimers, tetramers, and octamers (11–13). For keratins, which are heteropolymers of a type I and a type II keratin, the first step of assembly is the formation of a heterodimer. The type III IF proteins (vimentin, desmin, glial fibrillary acidic protein, and peripherin) which self-assemble in vitro as well as in vivo, form homodimers in the first step of polymerization, although heterodimers between different type III IF proteins can also be formed (14–15). As to the NFTPs, there is evidence for the existence of tetramers that contain mixtures of NFL and NFM (13); however, there is not yet any conclusive evidence as to the identity of the first-formed dimer. This dimer can be a homodimer, a heterodimer, or both. In the case of NFL, which can self-assemble in vitro, one would expect that it is able to form homodimers. In the formation of copolymers of NFL and NFM (or NFH), either homodimers of NFL (or NFH) or heterodimers of NFL/NFM (or NFL/NFH) could be formed, which could then interact with NFL homodimers to form filaments.

Although there is no direct evidence indicating that NFL/NFM or NFL/NFH heterodimers can be formed, the results of an antibody labeling study of bovine neurofilaments suggested the possible formation of heterodimers of NFL with NFM or NFH (16). This study showed that an epitope in the NFL tail, which was accessible in NFL homopolymers, was masked in native filaments. This phenomenon can be explained by the hypothesis that NFL/NFM and NFL/NFH heterodimers are the initial building blocks in native NFs. The elucidation of this first step of NFTP polymerization is important because it will help explain the detailed arrangement of the protein chains within the neurofilament.

To determine if the NFTPs preferentially form homodimers or heterodimers, one would like to be able to compare the relative affinities of an NFTP monomer for its own kind and for other NFTP monomers. However, these studies are confounded by the difficulty of stopping the polymerization at the dimer-
ization step. In order to circumvent this problem, we have employed the yeast two-hybrid system to measure the relative strengths of the NFTPs to interact with their own kind and with each other, as well as with vimentin.

**EXPERIMENTAL PROCEDURES**

Transformation and Manipulation of Yeast—For each transformation, yeast strain SFY526 (MATα, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can1, gal4–542, gal80–538, URA3) and GAL4-IacZ was grown and manipulated according to the MATCHMAKER Two-Hybrid System protocol (Clontech). Briefly, the two-hybrid vectors were co-transformed into the yeast cells by lithium acetate method, and the transformants were selected on the minus Trp-Leu synthetic dropout synthetic plates for 4 days at 30 °C.

Construction of Plasmids—All cDNAs were cloned into plasmids, pGBT9 and pGAD424 (Clontech), using standard molecular biology techniques (17). The plasmid constructs, pGBT-NFL24–542 and pGAD-NFL24–542 (generously provided by Dr. S. Chin, Columbia University), respectively encoded for the GAL4 DNAbinding and activation domain fused to the rat NFL from amino acid 24 to 542. To construct pGBT-NFL–415 and pGAD-NFL–415, the 2-kb SmaI–BamHI fragment was replaced by the 1-kb SmaI–KpnI fragment of pGBT-NFL24–542. To construct pGBT-NFM and pGAD-NFM, the 2.6-kb blunted EcoRI fragment containing the full-length rat NFM cDNA was isolated from pNFM2D (18) and was inserted at the EcoRI-digested ends by Klenow. The new resulting linearized plasmid DNAs were purified and blunted before setting up for self-ligation. The pGBT-NFL1–415 was made by replacing the 1-kb SmaI–KpnI fragment of pGBT-NFL24–542 with the 1.1-kb EagI (blunted)-KpnI fragment of pRSVII-NFL (8). The 1.3-kb EcoRI-BamHI fragment of the resulting pGBT-NFL1–415 construct was then cloned into pGAD424 to create pGAD-NFL1–415. To clone pGBT-NFM and pGAD-NFM, the 6.5-kb EcoRI fragment containing the full-length rat NFM cDNA was isolated from pNFM2D (18) and was inserted at the SmaI site of the pGBT9 and pGAD424. For construction of pGAD-NFM1–421 and pGAD-NFM1–421, the 1.2-kb SacI-HindIII (blunted) fragment from M1–421 (6) was used to replace the 2.5-kb SacI-BamHI (blunted) fragment of pGBT-NFM and pGAD-NFM. In order to create another frame for cloning, pGBT9 and pGAD424 were allowed to self-ligate after filling in the EcoRI-digested ends by Klenow. The new vectors were designated as pGBT9 and pGAD424. To make pGAD-NFM1–421 and pGAD-NFM1–421, the 1.2-kb SacI-HindIII (blunted) fragments of pGBT-NFM–421 and pGAD-NFM–421 were ligated to SmaI-digested pGBT9 and pGAD424. For cloning of pGBT-NFH and pGAD-NFH, the 3.4-kb NalI (blunted)-BamHI fragment encoding the entire rat NFH was isolated from pGEM3-NFH (8) and ligated to the SacI-BamHI-digested pGBT9 and pGAD424. To obtain the pGBT-NFH1–415 and pGAD-NFH1–415, the 2-kb Thh111I-BamHI fragments were removed from pGBT-NFH and pGAD-NFH; the linear plasmid DNAs were purified, blunted, and set up for self-ligation. To generate pGBT-NFL-Vim, the Bst111I (blunted)-BamHI fragment of pGEM-Vim (6) carrying the rat vimentin cDNA was cloned into the SacI-BamHI-digested pGBT9 and pGAD424. All plasmids were sequenced to ascertain reading frame with Sequenase 2.0 (U.S. Biochemical Corp.) according to the manufacturer’s protocols.

β-Galactosidase Assays—Interactions of proteins in the two-hybrid system can be monitored both qualitatively and quantitatively. Filter lift assays were performed for the qualitative measurement of β-galactosidase activity. Transformed yeast colonies were patched onto filters layered over minus Trp-Leu synthetic dropout synthetic agar plates overnight at 30 °C. Filters were submerged in liquid nitrogen for 10 s and placed on other filters that were presoaked in 5-bromo-4-chloro-3-indolyl β-D-galactoside solution containing 100 mM phosphate buffer (pH 7.0), 10 mM KCl, and 1 mM MgSO4. Filters were then incubated at 30 °C and periodically examined for the appearance of blue colonies. Quantitative assays were performed on liquid cultures. Cultures of 2.0 ml were grown to mid-log phase in minus Trp-Leu media. Cells were spun and resuspended into 0.5 ml of 100 mM phosphate buffer (pH 7.0), 10 mM KCl, and 1 mM MgSO4. Filters were then incubated at 30 °C for 1–2 h and finally quenched by adding 0.25 ml of 1 M Na2CO3. A β-galactoside assay kit (19) was used to measure the β-D-galactoside activity of transformed yeast colonies, and the results are summarized in Table I. None of the NFTP-containing chimeric proteins showed nonspecific activation of the LacZ reporter gene. β-Galactosidase activity could readily be detected when GAL4 transcriptional activation hybrids of NFL24–542 were co-transformed with GAL4 DNA binding hybrids of NFL24–542, NFM, and NFH, indicating the interactions of NFL withNFL, NFM, and NFH. Interestingly, even though both NFH and NFM contain rod domains that potentially interact, they did not interact with themselves or with each other in the two-hybrid system.

When the hybrid combinations for NFL, NFM, and NFH were reversed, i.e. GAL4 transcriptional activation hybrids of NFM or NFH were co-transformed with GAL4 DNA binding hybrids of NFL24–542, no β-galactosidase activity could be detected on filters. In order to determine whether this inconsistency was due to the lack of expression or improper folding of the activation domain-fused NFM and NFH, we also tested for their interactions with vimentin. We reasoned that since each of the NFTPs co-assembles with vimentin in transfected fibroblasts (9, 10), it was possible that these proteins would interact to form a heterodimer with vimentin in the yeast two-hybrid system. As shown in Table I, all of the NFTPs hybrids interacted with vimentin, although stronger interactions were detected when vimentin was expressed as a hybrid with the GAL4 DNA binding domain. The discrepancy of the results due to
switching the vectors may indicate that the GAD-vimentin hybrid is less well expressed than the GBT-vimentin hybrid. A lower level of expression of GAD-vimentin could also account for the apparently stronger interactions between GBT-vimentin and the GAD-NFTP hybrids than between GBT-vimentin and GAD-vimentin. In any case, our results imply that interactions of NFL with NLF, NFM, and NFH can occur, and in addition each of the NFTPs can interact with vimentin. The lack of interactions between NFM and NFH with themselves and the interactions observed between the GAD-NFL hybrid with the GBT-NFM and GBT-NFH hybrid are consistent with the tentative conclusion that NFL/NFM andNFL/NFHet- erodimers are formed in preference to NFM/NFM and NFH/ NFH homodimers; however, this conclusion is tempered, because GBT-NFL did not interact with GAD-NFM or GAD-NFH.

One problem with the previous results may be that the large size of the NFH and NFM tails in the hybrids may interfere with the activation of transcription of the lacZ reporter gene resulting in the lack of detection of their self-interactions by the β-galactosidase assay. To eliminate this possibility, we constructed tailless NFTPs in pGBT9 and pGAD424 to characterize all the possible interactions in dimer formation between the NFTPs. The tailless NFL (LΔ) and NFH (HΔ) constructs contained cDNA of rat NFL and NFH from amino acids 1 to 415 and the tailless NFM construct (MΔ) coded for amino acids 1–421. All the different combinations of the tailless constructs were co-transformed into yeast cells, and the interactions were monitored both by the filter assays and the liquid β-galactosidase assays. Yeast that had been co-transformed with combinations of hybrids containing NFTPs turned blue quickly after being permeabilized on filters, indicating that the tailless constructs interacted more strongly than the full-length hybrid (Fig. 1) and that lower β-galactosidase activities were observed when the ΔMΔ construct is fused to the GAL4 activation domain than when it is fused to the binding domain. However, the interactions between tailless NFL (LΔ) with ΔMΔ are stronger than the self-interaction of ΔMΔ regardless of which vector the NFM construct was cloned (compare ΔMΔ/LΔ and LΔ/ΔMΔ with ΔMΔ/ΔMΔ in Fig. 2).

In a recent study of the interactions between different domains of vimentin using the yeast two-hybrid system, Meng et al. (22) showed that vimentin dimers and tetramers both exist in the transformed yeast cells. This result raises the possibility that tetramers are also formed in our system and that part of the β-galactosidase activity of yeast co-transformed with NFL and NFM (or NFL and NFH) is due to the interactions between NFL homodimers and NFM (or NFH) homodimers. However, our results show that the self-interactions of NFL and NFH are weak (Figs. 1 and 2). Therefore, if tetramers are formed in this system, they are more likely to consist of either two NFL/NFM or NFL/NFH heterodimers or an NFL/NFL homodimer and an NFL/NFM (or NFL/NFH) heterodimer rather than NFL/NFL and NFL/NFM (or NFL/NFH) homodimers.

Overall, the results from the two-hybrid system show that the interactions between NFL and NLF, NFL and NFH, and

### Table II

| pGAD424   | pGBT9  |
|-----------|--------|
| HΔ        | +++    |
| MΔ        | +++    |
| LΔ        | ++++   |

Interactions between tailless NFTPs on β-galactosidase filter assays
The numbers of plus signs are comparable to those in Table I. HΔ, MΔ, and LΔ stand for the tailless NFTP constructs containing NFH1–415, NFM1–421, and NFL1–415, respectively.

![Fig. 1. Quantitative analysis of interactions between tailless neurofilament triplet proteins.](http://www.jbc.org/)
also very likely NFL and NFM are stronger than the interactions of NFM with NFM, NFH with NFH, or NFH with NFM. We therefore would predict that in the presence of all three NFTPs, these higher interaction affinities would cause NFL to self-polymerize and co-polymerize with NFM and NFH into filaments, whereas NFM and NFH would be less likely to form homopolymers or NFM/NFH heteropolymers than NFM/NFL or NFL/NFH heteropolymers. These results are therefore consistent with the ability of NFL to self-polymerize in vitro but are in apparent contradiction with the transfection results which show that NFL alone does not assemble into filaments in transfected cells (9, 10). However, the data are consistent with the results that showed that NFL is present only in the Triton X-100-insoluble fraction of transfected cells, even in the absence of a filamentous network, whereas relatively higher amounts of NFM and NFH are present in the soluble fraction (9). Thus NFL is able to form higher order filamentous structures, but not a filamentous network, which would require NFM and NFH (in the form of NFL/NFM and NFL/NFH heterodimers) to interact with the NFL homodimers.

Our results also show that the NFTPs are able to interact with vimentin in the two-hybrid system and imply that in transfected cultured cells they could co-polymerize with vimentin via the formation of heterodimers. Finally, our results suggest that during the assembly process of neurofilaments, both homodimers and heterodimers of the various NFTPs are present, but for NFM and NFH, heterodimer formation with NFL would be more preferred to formation of homodimers.

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REFERENCES

1. Fliegner, K. H., and Liem, R. K. H. (1991) Int. Rev. Cytol. 131, 109–167
2. Lee, M. K., and Cleveland, D. W. (1994) Curr. Opin. Cell Biol. 6, 34–40
3. Steinert, P. M., and Roop, D. R. (1988) Annu. Rev. Biochem. 57, 593–629
4. Gessler, N., and Weber, K. (1981) J. Mol. Biol. 151, 565–571
5. Liem, R. K. H., and Hutchison, S. B. (1982) Biochemistry 21, 3221–3226
6. Chin, S. S. M., and Liem, R. K. H. (1989) Eur. J. Cell Biol. 50, 475–490
7. Monteiro, M. J., and Cleveland, D. W. (1989) J. Cell Biol. 108, 579–593
8. Chin, S. S. M., and Liem, R. K. H. (1990) J. Neurosci. 10, 3714–3726
9. Ching, G. Y., and Liem, R. K. H. (1993) J. Cell Biol. 122, 1323–1336
10. Lee, M. K., Xu, Z., Wong, P. C., and Cleveland, D. W. (1993) J. Cell Biol. 122, 1337–1350
11. Quinlan, R. A., Hatzfeld, M., Franke, W. W., Lustig, A., Schulthess, T., and Engel, J. (1986) J. Mol. Biol. 192, 233–349
12. Hisanaga, S. I., and Hirokawa, N. (1990) J. Mol. Biol. 211, 871–882
13. Steinert, P. M., Idler, W. W., Cabral, F., Gottesman, M. M., and Goldman, R. D. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3692–3696
14. van den Heuvel, R. M. M., van Eys, G. J., van Gelder, L., and Cuypers, H. T. M. (1987) J. Cell Biol. 88, 475–482
15. Mulligan, L., Balin, B. J., Lee, V. M.-Y., and (1991) Struct. Biol. 106, 145–160
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. napoli, E. W., Chin, S. S. M., Colman, D. R., and Liem, R. K. H. (1987) J. Neurosci. 7, 2590–2599
18. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Fields, S., and Song, O. (1989) Nature 340, 245–247
20. Ching, G. Y., and Liem, R. K. H. (1993) J. Cell Biol. 122, 1323–1336
21. Meng, J., Khan, S., and Ip, W. (1996) J. Biol. Chem. 271, 1399–1604
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