Journal of Cell Science 109, 2493-2498 (1996)
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JCS8034

Neurofilament triplet protein interactions: evidence for the preferred formation of NF-L-containing dimers and a putative function for the end domains

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SUMMARY

In this report we examine the molecular interactions that lead to formation of neurofilaments, the intermediate filaments in neurons. Using the yeast two-hybrid system, we found that the rod domains of all three NF triplet proteins interacted strongly with one another and with rod domains of the Type III IF proteins, vimentin and desmin. A slight preference toward NF-L-containing dimers was observed over ones not containing NF-L. Interactions among the full length NF triplet proteins exhibited more specificity. Full length NF-L had only a relatively weak interaction with another full length NF-L molecule, but reacted more robustly with full length NF-M or NF-H lacking only part of the head domain. No homologous or heterologous dimerization of NF-M and NF-H was detectable. These results support the hypothesis that neurofilaments are obligate heteropolymers and that heterodimeric subunits are the preferred building blocks. They further suggest that the mechanism that specifies heterodimeric interaction among the NF triplet proteins resides in the end domains.

Key words: Cytoskeleton, Intermediate filament, Protein-protein interaction

INTRODUCTION

Neurofilaments (NFs) are major constituents of the neuronal cytoskeleton. They are found in neurons of both the central and peripheral nervous systems, and perturbation in their organization or expression has been correlated with neurologic disorders. Ectopic expression of NF subunits in transgenic mouse models results in phenotypes similar to those found in motor neuron disease and amyotropic lateral sclerosis (ALS) (Cote et al., 1993; Xu et al., 1993). A naturally occurring Japanese quail mutant that lacks NF-L also expresses motor neuron defects (Mizutani et al., 1992; Ohara et al., 1993).

The NF triplet proteins, NF-L, NF-M and NF-H, and α-internexin comprise the Type IV intermediate filament (IF) proteins (reviewed by Fuchs and Weber, 1994). All four conform to the tripartite structure typical of IF proteins, with a central α-helical rod domain flanked by amino-terminal head and carboxy-terminal tail domains. However, NFs are unique in several regards. First, in a given NF, there can be as many as four subunit proteins: NF-L, NF-M, NF-H and α-internexin or peripherin (Parysek et al., 1991), whereas other IFs are either homopolymers, or heteropolymers consisting of two polypeptides. Second, they are the only IFs to possess 4-6 nm wide sidearms which, in axons, span the intracellular space between filaments, appearing to cross-link them together (Hisanaga and Hirokawa, 1988). Because of this specialization, NFs often appear parallel to one another and to the long axis of the axon, and for this reason, they are widely believed to be the structural basis for the extended morphology of axons. The near-constant separation between NFs also has led Hoffman and coworkers to propose that they are a determinant of axonal caliber in large myelinated axons (Hoffman et al., 1987). Third, the NF triplet proteins possess carboxy-terminal tail domains that differ greatly in size and type of sequence motifs. All three NF proteins contain one or more glutamic acid-rich regions. NF-M and NF-H have atypically long tail domains containing multiple lysine-serine-proline (KSP) repeats, which are thought to be important phosphorylation sites. The tail domains of these two polypeptides are therefore expected to be highly charged, and are believed by some investigators to provide repulsive forces that maintain NFs in an axon at finite separations.

From early in vitro assembly studies, we know that NF-L readily assembles by itself into polymers in vitro (Geisler and Weber, 1981; Heins et al., 1993), NF-M does so only under highly unphysiological conditions, and NF-H does not do so at all (Gardner et al., 1984; Tokutake et al., 1984). However, both NF-M and NF-H form copolymers with NF-L that resemble naturally occurring NFs (Hisanaga and Hirokawa, 1988). Among IFs, the assembly of NFs presents a unique problem: what is the stoichiometry of neuronal IF proteins in the NF, particularly in the context of the commonly acknowledged schema of dimers and tetramers? On the basis of immunoelectron microscope evidence, we previously proposed that, in NFs containing only the triplet polypeptides, the basic unit of NF assembly is an NF-L containing dimer (Mulligan et al., 1991). In vivo evidence supporting this model, obtained by DNA transfection studies, has also been

reported by Ching and Liem (1993) and by Lee et al. (1993). More recently, Cohlberg et al. (1995) presented biochemical evidence for the existence in solution of heterotetramers containing either NF-L and NF-M, or NF-L and NF-H. However, neither the transfection studies nor the biochemical experiments directly addressed the question of the composition of the NF dimer in a tetramer. In this paper, we describe our recent studies on this problem, utilizing the yeast two-hybrid system to identify the protein-protein interactions involved in NF assembly. This approach allowed us to discriminate between homo- and heterodimerization by directly assaying for interactions between pairs of NF triplet proteins in a cellular context. Our results support the hypothesis that NFs are obligate heteropolymers, and demonstrate that the specificity of dimerization resides in the end domains of the NF triplet proteins.

MATERIALS AND METHODS

Yeast two-hybrid constructs

The full length murine NF-L (Gill et al., 1990) and rat NF-H cDNA were gifts from Dr Mervyn Monteiro (University of Maryland). The latter was derived from a full length clone originally reported by Chin and Liem (1990) and encoded a truncated NF-H protein that spanned part of the head together with the rod and tail domains in their entirety (residues 69-1,072). It was supplied to us in a modified pATH vector (Mercy et al., 1992) and could not be excised from it readily with restriction enzymes. Efforts to obtain the full length NF-H by the polymerase chain reaction (PCR) were unsuccessful; consequently a cDNA encoding a partial polypeptide was constructed for the two-hybrid experiments (see below). The full length rat NF-M cDNA (Napolitano et al., 1987) was a gift from Dr Ronald K. H. Liem (Columbia University). Full length clones of murine vimentin and desmin in pPC86 and pPC97 were gifts from Dr Jin-jun Meng of this laboratory (Meng et al., 1996).

Yeast vectors pPC86 and pPC97 containing, respectively, the GAL4 trans-activation (TA) domain, and the GAL4 DNA-binding (DB) domain (Chevray and Nathans, 1992), and the plasmid pMA431, containing the full length GAL4 cDNA, were gifts from Dr Jun Ma (Children's Hospital Research Foundation, Cincinnati, OH). The latter was used as a positive control for transformation. Two-hybrid constructs were made starting with the NF protein-encoding cDNAs described above. The rod domain constructs were made using PCR primers that began just 5′ and 3′ to the cDNA sequence that corresponded to the rod domains in the translated proteins; these are listed in Table 1. The full length NF-L and NF-M constructs were made using primers starting just 5′ and 3′ to the respective start and stop codons of the cDNAs (Table 2). All 5′ primers used contained a *Sal*I restriction site at their extreme 5′ ends, and all 3′ primers contained an *Eco*RI site at their extreme 5′ ends, so that the desired insert could be released from the PCR products with *Sal*I/*Eco*RI and cloned directly into the *Sal*I/*Eco*RI site of the yeast vector, pPC86. Once a construct was made in pPC86, the corresponding pPC97 construct was made by excising the insert from the pPC86 plasmid by *Sal*I/*Not*I digestion and ligated into the corresponding site of pPC97.

A construct encoding part of the head plus the entire rod and tail of NF-H (NF-H.rt), was assembled as follows. An *Nco*I/*Eco*RI fragment spanning part of the rod and the entire tail domain was excised from the NF-H cDNA from Dr Monteiro. The NF-H rod construct in pPC86 obtained by PCR cloning (see above) was also digested with *Nco*I/*Eco*RI and the rod-tail fragment was inserted into this site. A list of all two-hybrid constructs used in this study appears in Table 3. All constructs were verified by dideoxy sequencing across the cloning sites.

Yeast transformation and assays for β-galactosidase

Transformation of yeast *Saccharomyces cerevisiae* strain PCY2 (MATα Δgal4 Δgal80 URA3::GAL1-LacZ lys2-801^{amber} his3-Δ200 trp1-Δ63 leu2ade2-101^{ochre}) (Sikorski and Hieter, 1989), X-gal and fluorometric determination of β-galactosidase activity, were carried out as described by Meng et al. (1996). Wherever possible, the transformation was repeated by interchanging inserts and vectors, to control for potential steric effects that could result from differences in spatial juxtaposition of the NF protein domain relative to the GAL4 domain when they were expressed as a fusion protein. Swapping of vectors and inserts in this manner resulted in differences in β-galactosidase activity of less than 15%, and never altered the strength of the interaction relative to others that were studied (see Results).

RESULTS

Controls

The two-hybrid system makes use of the fact that the yeast transcription activator, GAL4, consists of two separate and essential domains (the DNA binding (DB) domain and the *trans*-activating (TA) domain) that must be in proximity to one another to function (Ma and Ptashne, 1987). To study the interaction between two proteins or protein domains, cDNAs encoding the proteins of interest are subcloned separately into two vectors such that they are expressed as fusion proteins with either the DB or TA domain (Fields and Song, 1989). Upon co-transformation of yeast with both plasmids, interaction between the fusion proteins of interest reconstitutes activity of the GAL4 protein, which then activates transcription of a *GAL1-lacZ* reporter gene.

An extensive series of controls for the particular vectors and yeast host we used has been presented in a recent report (Meng et al., 1996). In brief, these controls establish that, in the absence of cDNA inserts encoding IF proteins, the two vectors, pPC86 and pPC97, do not reconstitute GAL4 activity when introduced by transformation into PCY2 cells either alone or together. The β-galactosidase activity that one measures after co-transformation with the constructs therefore truly represents the level of interaction between the proteins or protein domains encoded by the constructs.

Table 1. Primers used to amplify the NF rod regions

Construct	5′-primer	3′-primer
NF-L.rod	5′-AATAAGTCGACGCTGCAGGACCTCAACCA-3′	5′-GATCTGAATTCCTGAGCCTGGTCTCTTC-3′
NF-M.rod	5′-AATAAGTCGACGCTGCAGGGGCTGAA-3′	5′-ATTAGAATTCATTGTGACTGAGGGCT-3′
NF-H.rod	5′-GGTAAGTCGACGCTAGACACCCTAA-3′	5′-ATTAGAATTCATCCGACACTCTTCGCCT-3′

Table 2. Primers used to amplify full length NF cDNA

Construct	5′-primer	3′-primer
NF-L.fl	5′-AATAAGTCGACCTCGTTCGGCTACGATCCGTA-3′	5′-GATCTGAATTCCATCAATCTTTCTTCTTA-3′
NF-M.fl	5′-AATAAGTCGACGATGAGCTACACGCTGGA-3′	5′-ATTAGAATTCTCTTAGTCACCCTGGGT-3′

A second important fact demonstrated by Meng et al. (1996) is that the two-hybrid system was highly specific in the context of IF protein interactions. This is also partly borne out in the present study. As shown in Table 4, the NF rod domains interacted strongly with the Type III IF proteins, vimentin and desmin (lines 8-13), but did not interact with the Type I keratin K18 and the Type II keratin K8 (lines 14 and 15). This is in complete agreement with what is known about the ability of IF proteins to form copolymers (Fuchs and Weber, 1994), and is consistent with the known co-expression of NF triplet proteins with vimentin in the nervous system during early development (Nixon and Shea, 1992). Thus, the suitability of the two-hybrid system for studying IF protein interactions also extends to the NF triplet proteins, described below.

NF rod domains interact indiscriminately with one another and with Type III IF rod domains

NFs are heteropolymers in vivo (Ching and Liem, 1993; Lee et al., 1993). The question remains, however, as to whether heteropolymers formed by the NF triplet proteins contain homodimers of NF-L, NF-M and NF-H, or heterodimers, as has been found in the case of the keratins (Hatzfeld and Weber, 1990). Prompted by the observation that differences in heptad spacing and overall length of rod domains play a key role in determining whether one IF protein can interact with another (Steinert et al., 1993), we first examined interactions between the NF triplet rod domains to look for evidence of homo- or heterodimerization. NF triplet protein rod domains were expressed pairwise in PCY2 cells by co-transformation and, after colonies could be discerned visually, plates were replicated onto nitrocellulose membrane and the filters stained with X-gal (Breeden and Nasmyth, 1985) to identify colonies in which two-hybrid interaction had taken place. Results from these co-transformations are presented in Table 4.

The known interaction between full length vimentin-GAL4 fusions (Meng et al., 1996) was used as a positive control (line 1). Also included were co-transformations in which the NF triplet rod domains were co-expressed with the Type III IF proteins, vimentin and desmin (lines 8-13). Vimentin is co-expressed with NFs during early development (Nixon and Shea, 1992), and desmin shares extensive homology with vimentin in the rod domain; thus we expected both to interact with NF rods. The NF-L rod was also co-transformed separately with the acidic keratin, K18, and with the basic keratin, K8 (lines 14-15); these combinations were included as negative controls, as interactions between NF triplets and keratin has never been reported.

With the exception of the NF-keratin co-transformations, all combinations listed in Table 4 produced significant levels of β-galactosidase activity. The colonies on the replica plate turned dark blue by the end of the one-hour incubation. The homophilic interaction between NF-L-GAL4 fusions (line 2, Table 4) was especially robust, as a color change was noted after 15 minutes, and by 30 minutes it was dark blue.

The surprising lack of selectivity of interactions amongst the NF rod domains was further examined by quantitative analysis of the β-galactosidase activity. After replica plating, colonies were picked from the plates and expanded in liquid culture. Homogenates were produced from harvested cells and used in a fluorometric assay using 4-methylumbelliferyl galactopyranoside (4-MUG) as substrate. Results from this quantitative analysis are also presented in Table 4 and graphically in Fig. 1. Overall, the homophilic interaction between NF-L rods (L/L in Fig. 1) was again the strongest. Somewhat weaker but nonetheless quite robust were the heterotypic interactions between rod domains of NF-L and either NF-M (L/M in Fig. 1) or NF-H (L/H in Fig. 1). β-galactosidase activities for NF-M/NF-M, NF-H/NF-H and NF-M/NF-H interactions were still lower. Nonetheless, they were significant. Thus, these quantitative results show that all three NF rod domains readily interact with one another but suggest a preference for NF-L-containing dimers.

The NF rod domains also interacted with full length versions

Table 3. Yeast two-hybrid constructs used in this study

GAL4 TA-domain vectors	*GAL4 DB*-domain vectors
pPC86 NF-L.rod	pPC97 NF-L.rod
pPC86 NF-M.rod	pPC97 NF-M.rod
pPC86 NF-H.rod	pPC97 NF-H.rod
pPC86 NF-L.fl	pPC97 NF-L.fl
pPC86 NF-M.fl	pPC97 NF-M.fl
pPC86 NF-H.rt	pPC97 NF-H.rt
pPC86 vimentin	pPC62 vimentin*
pPC86 desmin	pPC97 desmin
pPC86 K8	pPC97 K8
pPC86 K18	pPC97 K18

*This construct was in pPC62, which was identical to pPC97 except for differences in restriction enzyme sites within the multiple cloning site.

Table 4. Two-hybrid co-transformations using NF rod domains

	Plasmids co-transformed		β-galactosidase activity (X-gal staining)	β-galactosidase activity (4-MUG assay)
	pPC86-	pPC97-		
1	Vimentin +	Vimentin*	+++	1947.2
2	NF-L.rod +	NF-L.rod	+++	1800.4
3	NF-M.rod +	NF-M.rod	+++	435.5
4	NF-H.rod +	NF-H.rod	+++	922.9
5a	NF-M.rod +	NF-H.rod	+++	1075.0
5b	NF-H.rod +	NF-M.rod	+++	1034.2
6a	NF-L.rod +	NF-M.rod	+++	1319.0
6b	NF-M.rod +	NF-L.rod	+++	1379.3
7a	NF-L.rod +	NF-H.rod	+++	1419.2
7b	NF-H.rod +	NF-L.rod	+++	1452.2
8a	NF-L.rod +	Vimentin*	+++	483.3
8b	Vimentin +	NF-L.rod	+++	542.6
9a	NF-M.rod +	Vimentin*	+++	163.9
9b	Vimentin +	NF-M.rod	+++	184.7
10a	NF-H.rod +	Vimentin*	+++	585.3
10b	Vimentin +	NF-H.rod	+++	632.2
11a	NF-L.rod +	Desmin	+++	636.0
11b	Desmin +	NF-L.rod	+++	614.1
12a	NF-M.rod +	Desmin	+++	314.2
12b	Desmin +	NF-M.rod	+++	363.7
13a	NF-H.rod +	Desmin	+++	338.8
13b	Desmin +	NF-H.rod	+++	363.7
14a	NF-L.rod +	K18	–	5.47
14b	K18 +	NF-L.rod	n.d.	n.d.
15a	NF-L.rod +	K8	–	0
15b	K8 +	NF-L.rod	n.d.	n.d.

Wherever possible (denoted by a and b entries), transformations were repeated with interchanged inserts and vectors. Intensity of X-gal staining is reported as +++ (dark blue), ++ (medium blue, clearly visible), and + (light blue, just visible). – denotes colorless colonies. Each data point is an average obtained from at least four independent positive colonies.

*This construct was in pPC62, which was identical to pPC97 except for differences in restriction enzyme sites within the multiple cloning site.

n.d., not determined.

of the Type III IF proteins, vimentin and desmin (lines 8-13, Table 4, and Fig. 2), although overall the levels of β-galactosidase activity produced by these interactions were lower than those produced by interactions involving only NF triplet rod domains. As expected, no interaction was observed between the NF-L rod and either full length keratins.

Note that in all cases, interchanging vectors and inserts in the co-transformations resulted in a difference of reporter gene activity of no more than 15% (Table 4, comparing the entries designated a versus those designated b). This renders unlikely the possibility that the enzyme levels were significantly affected by inherent problems imposed by the system itself. Further, the trend of reporter gene activity in a given series of transformations, e.g. lines 3-7 in Table 4, was never altered by swapping vectors, suggesting that it is a reliable indicator of the degree of interaction among different pairs of NF triplets.

Two-hybrid interactions between full length neurofilament triplet polypeptides

Given that the rod domains of the NF triplet proteins interacted indiscriminately with one another and with vimentin and desmin, we next examined if the selective nature of NF interactions observed in transfected cells (Ching and Liem, 1993; Lee et al., 1993) and in solution (Cohlberg et al., 1995) might reside in the end domains. This was done by transforming pairwise into PCY2 cells cDNAs encoding full length NF-L and NF-M, and the rod/tail domain of NF-H. Qualitative and quantitative results from these transformations are presented in Table 5 and Fig. 3.

Several significant results emerged from this series of co-transformations. First, interactions among full length NF triplet polypeptides exhibited a degree of selectivity not seen in rod domain interactions. Both X-gal staining of lifts and the 4-MUG assay corroborated that certain combinations of NF triplets produced significant levels of β-galactosidase activity, while others clearly did not. Second, interactions in which one member of the pair was full length NF-L were favored. The formation of NF-L/NF-M and NF-L/NF-H heterodimers producing the highest levels of β-galactosidase activity (lines 5-6, Table 5 and Fig. 3) whereas a low but significant level was observed when full length NF-L was allowed to form homodimers (line 4, Table 5, and Fig. 3). Dimerization between NF-M and NF-H evidently occurred only to a very limited extent, as co-transformations of NF-M- and NF-H-expressing plasmids did not produce detectable levels of enzyme activity

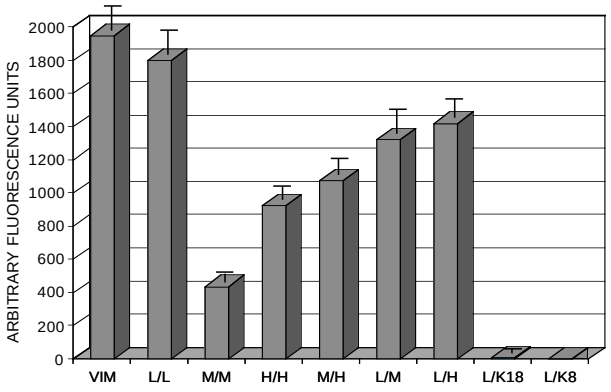

Fig. 1. Quantitative two-hybrid analysis of interactions among rod domains of NF triplet polypeptides. The extent of interaction is expressed as β-galactosidase activity, obtained in a fluorescence assay as described in Materials and Methods. Each value shown in the figure is an average of enzyme activity of at least four independent positive colonies. Along the *x*-axis: VIM, full length vimentin in both two-hybrid vectors; L/L, NF-L rod inserts in both vectors; M/M, NF-M rod inserts in both vectors; H/H, NF-H rod inserts in both vectors; M/H, NF-M rod insert in pPC86, NF-H rod insert in pPC97; L/M, NF-L rod insert in pPC86, NF-M rod insert in pPC97; L/H, NF-L rod insert in pPC86, NF-H rod insert in pPC97; L/K18, NF-L rod insert in pPC86, full length K18 in pPC97; L/K8, NF-L rod insert in pPC86, full length K8 in pPC97. Data from the reciprocal transformations (vectors and inserts interchanged) are presented in Table 4 but not in this figure. Differences resulting from such swapping are insignificant. Error bars denote standard error of the mean (s.e.m.).

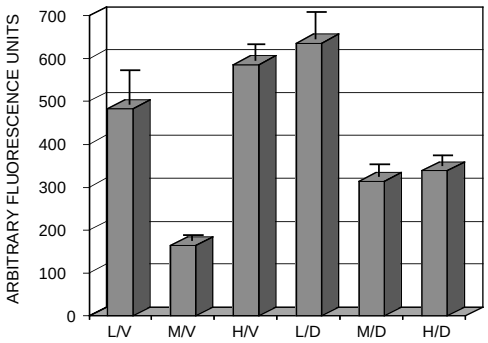

Fig. 2. Quantitative two-hybrid analysis of interactions between NF triplet rod domains and full length vimentin and desmin. Each value shown in the figure is an average of β-galactosidase activity of at least four independent positive colonies. Along the x-axis, L/VIM, M/VIM and H/VIM denote transformations in which NF-L, NF-M or NF-H rod insert was in pPC86, and full length vimentin was in pPC62, a vector identical to pPC97 except for the presence of different restriction sites within the multiple cloning site. Similarly, L/DES, M/DES and H/DES denote transformations in which NF-L, NF-M or NF-H rod insert was in pPC86 and full length desmin was in pPC97. Data from the reciprocal transformations (vectors and inserts interchanged) are presented in Table 4 but not in this figure. Differences resulting from such swapping are insignificant. Error bars denote standard error of the mean (s.e.m.).

Table 5. Two-hybrid co-transformations using full-length NF triplets

	Plasmids co-transformed	β-galactosidase activity	β-galactosidase activity
	pPC86- pPC97	(X-gal staining)	(4-MUG assay)
1	NF-M.fl + NF-M.fl	–	1.5
2a	NF-M.fl + NF-H.rt*	–	2.3
2b	NF-H.rt + NF-Mfl	–	2.2
3	NF-H.rt + NF-H.rt	–	14.0
4	NF-L.fl + NF-L.fl	+	72.1
5a	NF-L.fl + NF-M.fl	++	192.8
5b	NF-Mfl + NF-L.fl	++	173.8
6a	NF-L.fl + NF-H.rt	++	233.3
6b	NF-H.rt + NF-L.fl	++	280.9

Wherever possible (denoted by a and b entries), transformations were repeated with interchanged inserts and vectors. Intensity of X-gal staining is reported as +++ (dark blue), ++ (medium blue, clearly visible), and + (light blue, just visible). – denotes colorless colonies. Each data point is an average obtained from at least four independent positive colonies.

*NF-H.rt encodes the complete rod plus tail domain of NF-H, but is missing 69 amino acids at the amino end of the head domain (see Materials and Methods).

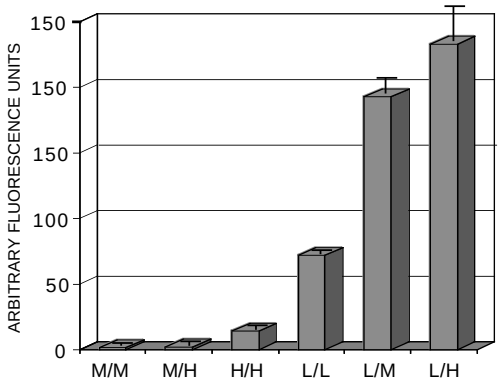

Fig. 3. Formation of NF-L-containing dimers is preferred over ones that do not contain NF-L. Quantitative two-hybrid analysis was carried out as described in the figure legends of Fig. 1 and Fig. 2 except that constructs encoding full length NF triplet polypeptides were used. Although the overall levels of enzyme activity are lower than in the case where rod domain constructs are used, the preferential formation of NF-L-containing dimers is clearly observed in the L/L, and particularly in the L/M and L/H transformations. Error bars denote standard error of the mean (s.e.m.).

(lines 1-3, Table 5). Again, interchanging vectors and inserts in co-transformations produced highly consistent results.

DISCUSSION

The identity and protein composition of the basic subunit of neuronal IFs are a complex issue. Six major IF proteins have been identified in neuronal IFs: the NF triplet polypeptides, α-internexin, peripherin and vimentin. Although all six have not been shown to be present in a single NF, peripherin has been colocalized with the NF triplets (Parysek et al., 1991), as have α-internexin (Ching and Liem, 1993) and vimentin (Nixon and Shea, 1992). Moreover, the expression of these proteins is regulated during development (Liem, 1993), so the protein composition of neuronal IFs is most likely very different at different developmental ages. Whether such differences are reflected in varying composition of the subunit is not known. Further, there have not been attempts to determine whether all neuronal IF proteins can in fact interact with one another to form dimers and tetramers, the commonly recognized subunits of IFs.

While NF-M and NF-H have long been known to be incapable of forming filaments under physiological conditions, the ability of NF-L to form filaments by itself in vitro (Geisler and Weber, 1981; Heins et al., 1993) was at first puzzling. The recent studies of Ching and Liem (1993) and Lee et al. (1993), however, demonstrated convincingly by DNA transfection that NF-L alone does not form IF networks when expressed in IF-free SW13 cl. 2 cells. Only when it was present together with either NF-M or NF-H did 10 nm filament networks arise. Thus, NFs are heteropolymers in vivo.

A question that has not been fully resolved is whether the assembly of NF involves a tetramer, as has been observed in other IFs, and if so, whether it is composed of heterodimers as in the case of the keratins (Hatzfeld and Weber, 1990), homodimers, or another, yet unidentified, configuration of polypeptides. The earlier work of Carden and Eagles (1983), utilizing chemical cross-linking, suggested that each triplet polypeptide was capable of forming homodimers, and that an NF-M/NF-H heterodimer was also possible. However, these conclusions were later revised (Carden and Eagles, 1986) to state that NF-L homodimers and NF-L/NF-M heterodimers were the prominent species. A partial resolution of this question came from the studies of Cohlberg et al. (1995) who, by hydrodynamic measurements, detected the existence of two species of heterotetramers, containing either NF-L and NF-M, or NF-L and NF-H. Interestingly, these investigators did not detect homotetramers of any composition, and whether the heterotetramers consist of homodimers of the composition L_2M_2 and L_2H_2, or heterodimers of the composition $(LM)_2$ and $(LH)_2$, remains unclear.

The present study examines the structure of the NF dimer by the yeast two-hybrid system (Fields and Song, 1989), a powerful approach which we showed in a previous study (Meng et al., 1996) to be particularly efficacious for examining interactions in IF assembly. An advantage of this approach over chemical cross-linking is that it only identifies polypeptides that interact with one another, whereas chemical cross-linking does not discriminate between interacting polypeptides and those that are merely sufficiently close to one another to allow cross-linking to take place. Our results provide strong support for the proposal that a heterodimer consisting of NF-L and either NF-M or NF-H is the preferred dimeric intermediate (Mulligan et al., 1991; Ching and Liem, 1993; Lee et al., 1993). Formation of NF-L homodimers is also possible but to a lesser extent in the context of the two-hybrid system, whereas dimers consisting of only NF-M and NF-H are not favored.

These conclusions are based on quantitative determinations of β-galactosidase activity, and formally we cannot rule out the possibility that the differences in enzyme activity arose from constraints imposed by the system itself. For example, it may be argued that the fusion protein formed by a given triplet protein with the DB domain of GAL4 may be structurally very different from the one it forms with the TA domain, so that the ability of the final complex to drive expression of lacZ may not be a reliable measure of strength of interaction. However, the fact that interchanging vectors and inserts in all of our experiments produced a <20% (<10% in most cases) change in β-galactosidase activity (Tables 4 and 5) would tend to argue against this possibility. The consistency of enzyme activity after vector swapping argues that all of our constructs functioned as expected, that steric problems were insignificant, and that the differences we detected likely reflect real differences in strength of protein-protein interaction.

It is also evident from our results that the end domains of the NF triplet polypeptides play a role in specifying heterotypic dimerization. Interactions between full length NF triplet polypeptides exhibit a high degree of preference toward the NF-L/NF-X dimer, where X can be either NF-M or NF-H. In contrast, rod domains of the NF triplet dimerize with one another strongly and indiscriminately; in fact, they dimerize readily with vimentin and desmin, though failing to interact with either keratin K8 or K18. We should note, however, that this conclusion is qualified by the fact that our 'full length' NF-H construct in fact encodes only the rod and tail domains and is missing 69 residues at the amino end of the head domain. Formally, therefore, our conclusion cannot be applied to the head domain of NF-H. As to how important the head domain of NF-H is to filament assembly in and of itself is not known, as this NF protein does not form filaments by itself under any conditions (Gardner et al., 1984).

It is noteworthy that interactions between NF triplet rod domains (Table 4) produced significantly higher levels of β-galactosidase activity than those between the corresponding full length proteins (Table 5). It may be argued that the full length triplet proteins are much larger than their respective rod domains and this might have placed the TA and DB domains of GAL4 sufficiently far apart in the final complex of NF/TA-NF/DB to partially compromise activation of the *lacZ* gene. This is unlikely, however, as IF protein dimerization is thought to take place in a parallel and in-register fashion (Parry et al., 1985), which would place the GAL4 domains on the same end of the complex. It follows that the distance between the GAL4 domains in the complex should be independent of the size of the NF protein. Two other explanations are more plausible. First, at least the NF-L rod is capable of forming paracrystalline arrays in vitro (Heins et al., 1993). Whether this occurs with NF-GAL4 fusion proteins in yeast is not known, but if it did, the two-hybrid reporter gene activity from rod domain co-transformations would probably reflect this interaction and therefore be artifactually inflated. Second, a more attractive, but speculative, explanation is that the end domains of the NF triplet play a role in specifying axial alignment of NF polypeptides during dimerization. In this scenario, full length polypeptides can dimerize only in one alignment, most likely a parallel and in-register one (Parry et al., 1985). In contrast, in the absence of end domains, the relative axial position of two rod domains is not specified and pairing can occur at any axial stagger so long as there is sufficient overlap to stabilize the dimer. This added latitude of interaction could lead to augmented β-galactosidase activity in the two-hybrid analysis. A detailed examination of the end domains of the NF triplet and their potential binding partners should provide further information regarding this question.

We are grateful to Drs Mervyn Monteiro (University of Maryland) and Ron Liem for supplying the neurofilament triplet cDNAs, Dr Jun Ma (Childrens Hospital Research Foundation) for providing pMA431 and the two-hybrid vectors, and Dr Jin-jun Meng of this laboratory for providing the desmin and vimentin two-hybrid constructs. We also thank Dr Meng for guidance in use of the quantitative β-galactosidase assay. This work was supported by USPHS grant RO1-AR35973 and in part by ACS.

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(Received 6 May 1996 – Accepted 16 July 1996)