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 amyotrophic 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 truncated NF-H was cloned from the PC86 plasmid by SalI/Ncol digestion and ligated into the corresponding site of pC97.

A construct encoding part of the head plus the entire rod and tail of NF-H (NF-Hr), was assembled as follows. An Ncol/EcoRI 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 pC86 obtained by PCR cloning (see above) was also digested with Ncol/EcoRI 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 dye-deoxy sequencing across the cloning sites.

**Yeast transformation and assays for β-galactosidase**
Transformation of yeast *Saccharomyces cerevisiae* strain PCY2 (MATa Δgal4 Δgal80 URA3::GAL1-LacZ lys2-801amber his3-Δ200 trp1-Δ63 leu2ade2-101cortr) (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 transactivation, 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 pC97, do not reconstitute GAL4 activity when introduced by transformation into PCY2 cells either alone or together. The β-galactosidase activity that one measures after transformation with the constructs therefore truly represents the level of interaction between the proteins or protein domains encoded by the constructs.

<table>
<thead>
<tr>
<th>Table 1. Primers used to amplify the NF rod regions</th>
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<tr>
<td>Construct</td>
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</tr>
<tr>
<td>NF-L.rod</td>
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<tr>
<td>NF-M.rod</td>
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<tr>
<td>NF-H.rod</td>
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<tr>
<th>Table 2. Primers used to amplify full length NF cDNA</th>
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<tr>
<td>Construct</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>NF-L.fl</td>
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<tr>
<td>NF-M.fl</td>
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</table>
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. 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.

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.

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

<table>
<thead>
<tr>
<th>GAL4 TA-domain vectors</th>
<th>GAL4 DB-domain vectors</th>
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<tbody>
<tr>
<td>pPC86 NF-L.rod</td>
<td>pPC97 NF-L.rod</td>
</tr>
<tr>
<td>pPC86 NF-M.rod</td>
<td>pPC97 NF-M.rod</td>
</tr>
<tr>
<td>pPC86 NF-H.rod</td>
<td>pPC97 NF-H.rod</td>
</tr>
<tr>
<td>pPC86 NF-L.fl</td>
<td>pPC97 NF-L.fl</td>
</tr>
<tr>
<td>pPC86 NF-M.fl</td>
<td>pPC97 NF-M.rod</td>
</tr>
<tr>
<td>pPC86 NF-H.rod</td>
<td>pPC97 NF-H.rod</td>
</tr>
<tr>
<td>pPC86 vimentin</td>
<td>pPC97 desmin</td>
</tr>
<tr>
<td>pPC86 desmin</td>
<td>pPC97 NF-L.rod</td>
</tr>
<tr>
<td>pPC86 K8</td>
<td>pPC97 K8</td>
</tr>
<tr>
<td>pPC86 K18</td>
<td>pPC97 K18</td>
</tr>
</tbody>
</table>

*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**

<table>
<thead>
<tr>
<th>Plasmids co-transformed</th>
<th>β-galactosidase activity (X-gal staining)</th>
<th>β-galactosidase activity (4-MUG assay)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Vimentin + Vimentin*</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>NF-L.rod + NF-L.rod</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>NF-M.rod + NF-M.rod</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>NF-H.rod + NF-H.rod</td>
<td>+++</td>
</tr>
<tr>
<td>5a</td>
<td>NF-M.rod + NF-H.rod</td>
<td>+++</td>
</tr>
<tr>
<td>5b</td>
<td>NF-H.rod + NF-H.rod</td>
<td>+++</td>
</tr>
<tr>
<td>6a</td>
<td>NF-L.rod + NF-M.rod</td>
<td>+++</td>
</tr>
<tr>
<td>6b</td>
<td>NF-M.rod + NF-L.rod</td>
<td>+++</td>
</tr>
<tr>
<td>7a</td>
<td>NF-L.rod + NF-H.rod</td>
<td>+++</td>
</tr>
<tr>
<td>7b</td>
<td>NF-H.rod + NF-L.rod</td>
<td>+++</td>
</tr>
<tr>
<td>8a</td>
<td>NF-L.rod + Vimentin*</td>
<td>+++</td>
</tr>
<tr>
<td>8b</td>
<td>Vimentin + NF-L.rod</td>
<td>+++</td>
</tr>
<tr>
<td>9a</td>
<td>NF-M.rod + Vimentin*</td>
<td>+++</td>
</tr>
<tr>
<td>9b</td>
<td>Vimentin + NF-M.rod</td>
<td>+++</td>
</tr>
<tr>
<td>10a</td>
<td>NF-H.rod + Vimentin*</td>
<td>+++</td>
</tr>
<tr>
<td>10b</td>
<td>Vimentin + NF-H.rod</td>
<td>+++</td>
</tr>
<tr>
<td>11a</td>
<td>NF-L.rod + Desmin</td>
<td>+++</td>
</tr>
<tr>
<td>11b</td>
<td>Desmin + NF-L.rod</td>
<td>+++</td>
</tr>
<tr>
<td>12a</td>
<td>NF-M.rod + Desmin</td>
<td>+++</td>
</tr>
<tr>
<td>12b</td>
<td>Desmin + NF-M.rod</td>
<td>+++</td>
</tr>
<tr>
<td>13a</td>
<td>NF-H.rod + Desmin</td>
<td>+++</td>
</tr>
<tr>
<td>13b</td>
<td>Desmin + NF-H.rod</td>
<td>+++</td>
</tr>
<tr>
<td>14a</td>
<td>NF-L.rod + K8</td>
<td>–</td>
</tr>
<tr>
<td>14b</td>
<td>K8 + NF-L.rod</td>
<td>n.d.</td>
</tr>
<tr>
<td>15a</td>
<td>NF-L.rod + K8</td>
<td>–</td>
</tr>
<tr>
<td>15b</td>
<td>K8 + NF-L.rod</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

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-

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; L/M, NF-M rod insert in pPC86, NF-H rod insert in pPC97; L/H, 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.).

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.

<table>
<thead>
<tr>
<th>Plasmids co-transformed</th>
<th>β-galactosidase activity (X-gal staining)</th>
<th>β-galactosidase activity (4-MUG assay)</th>
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<tbody>
<tr>
<td>pPC86-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPC97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NF-M.fl + NF-M.fl</td>
<td>–</td>
</tr>
<tr>
<td>2a</td>
<td>NF-M.fl + NF-H.rt*</td>
<td>–</td>
</tr>
<tr>
<td>2b</td>
<td>NF-H.rt + NF-M.fl</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>NF-H.rt + NF-H.rt</td>
<td>–</td>
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<tr>
<td>4</td>
<td>NF-L.fl + NF-L.fl</td>
<td>+</td>
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<tr>
<td>5a</td>
<td>NF-L.fl + NF-M.fl</td>
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<td>5b</td>
<td>NF-M.fl + NF-L.fl</td>
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<td>6a</td>
<td>NF-L.fl + NF-H.rt</td>
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<tr>
<td>6b</td>
<td>NF-H.rt + NF-L.fl</td>
<td>++</td>
</tr>
</tbody>
</table>

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).*

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

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chemical cross-linking, suggested that each triplet polypeptideimers, or another, yet unidentified, configuration of polypep-
in the case of the keratins (Hatzfeld and Weber, 1990), homod-
other IFs, and if so, whether it is composed of heterodimers as
NFs are heteropolymers in vivo.

Despite the absence of NF-L in the free SW13 cl. 2 cells. Only when it was present together with
NF-L alone does not form IF networks when expressed in IF-
however, demonstrated convincingly by DNA transfection that
recent studies of Ching and Liem (1993) and Lee et al. (1993),
and Weber, 1981; Heins et al., 1993) was at first puzzling. The
incapable of forming filaments under physiological conditions,
L 2 M 2 and L 2 H 2 , or het-
erodimers 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 polypep-
tides 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 interme-
diate (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 we detected likely reflect real differences
in strength of protein-protein interaction. However, the
fact that our ‘full length’ NF-H construct in fact encodes only the
head domain of NF-H is to filament assembly in and of itself is
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 exper-
iments produced a <20% (<10% in most cases) change in
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 func-
tioned 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 polypep-
tides 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).

Some graphical and tabular data accompany the text.

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 com-
position 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), homod-
imers, or another, yet unidentified, configuration of polypep-
tides. 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 Coihberg et al. (1995) who, by hydrodynamic mea-
surements, detected the existence of two species of heterote-
tramers, 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 L2M2 and L2H2, or het-
erodimers of the composition (LM)2 and (LH)2, remains unclear.

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.
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 artificially 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.

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REFERENCES


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