STUDY OF *IN VIVO* AND *IN VITRO* ASSOCIATIONS BETWEEN NEURONAL INTERMEDIATE FILAMENT PROTEINS

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ABSTRACT

The developmental profile of the neurofilament (NF) triplet proteins, α-internexin and peripherin in cultured embryonal dorsal root ganglion neurons was determined by Western blot analysis. At day 0 in culture, the neurons contained mostly α-internexin. A significant increase in peripherin levels was seen at days 1-2, in the mid-sized (NFM) and low molecular weight (NFL) NF subunits at days 2-3, and in the high molecular weight (NFH) NF subunit at days 5-6. Immunofluorescence microscopy showed that the five neuronal intermediate filament (nIF) proteins were colocalized in all neuronal cell bodies and neurites. Analysis of Triton X-100-soluble extracts from okadaic acid-treated DRG cultures revealed that peripherin and α-internexin followed the same fragmentation pattern observed for NFs. When these extracts were incubated with antibodies specific for the individual proteins, co-immunoprecipitation of NFH with NFL, NFM with NFL, NFM with α-internexin, and α-internexin with peripherin were observed, demonstrating that nIF proteins in cultured sensory neurons form a highly integrated network.

Formation of protein dimers involving α-internexin, peripherin, NFH, NFM and NFL, was then investigated by partial renaturation of various combinations of individually purified subunits in buffered 2 M urea. Oligomers that were formed were resolved by "blue" native electrophoresis (BN-PAGE) modified to include urea in the polyacrylamide gels. Combining this method with Western blot analysis, disulfide cross-linking and SDS-polyacrylamide gel electrophoresis in the second dimension showed that with the exception of NFH/NFM mixtures where these proteins remained monomeric, all pairwise combinations of nIF proteins resulted in homo- and heterodimer formation. However, heterodimer formation between α-internexin and peripherin could neither be demonstrated nor ruled out.

BN-PAGE with a lateral urea concentration gradient further indicated that posttranslational modifications present on spinal cord NFL and presumably lacking in the bacterially expressed protein, destabilized its homodimeric interactions in favor of heterodimeric interactions with NFH and NFM, perhaps to ensure proper dimer stoichiometry for NF assembly *in vivo*. A mutation in NFL suspected of interfering with NF assembly in motor neurons was also shown to destabilize NFL-containing dimers indicating that this mutation impacts on the earliest stage of NF assembly. This work provides the first direct biochemical assesment of nIF protein dimer formation and stability.

RÉSUMÉ

L'expression des trois protéines de neurofilaments (NF) NFL, NFM et NFH ainsi que l'α-internexine et la périphérine a été déterminée par immunobuvardage "Western" durant le développement de neurones embryonnaires de ganglions dorsaux en culture. Au jour 0 de la culture, les neurones contenaient surtout de l'α-internexine. La quantité de périphérine a augmenté de façon significative après 1-2 jours en culture, celle de NFL et NFM après 2-3 jours, et celle de NFH après 5-6 jours. L'analyse des cultures par immunofluorescence indirecte a révélé que les cinq protéines de filament intermédiaire neuronaux (FIn) étaient co-localisées dans tout les compartiments des neurones. L'analyse d'extraits cellulaires solubles au Triton X-100 suite à un traitement à l'acide okadaïque a indiqué que la solubilisation de l'α-internexine et de la périphérine suivaient le même profile que pour NFL, NFM et NFH. L'incubation de ces extraits cellulaires avec des anticorps spécifiques pour chaque protéine permit la co-immunoprécipitation de NFH avec NFL, NFM avec NFL, NFM avec α-internexine et α-internexine avec périphérine démontrant ainsi que ces cinq protéines sont associées dans le réseau de FIn des neurones sensorielles en culture.

Suite à leur purification individuelle, la renaturation partielle de différentes paires de protéines de FIn dans un tampon contenant de l'urée à 2 M, a permis la formation d'oligomères qui furent analysés par électrophorèse native "bleue" (BN-PAGE), modifiée de façon à inclure la même concentration d'urée dans les gels de polyacrylamide. Combinée à l'immunobuvardage "Western", à la création de liens dissulphures et à l'électrophorèse sur gel de polyacrylamide en présence de SDS, cette méthode a démontré la formation d'homo- et d'hétérodimères entre toutes les paires de protéines de FIn, à l'exception de NFH et NFM qui restèrent à l'état monomérique. Il fut cependant impossible de démontrer la présence et/ou l'absence d'hétérodimères entre l'α-internexine et la périphérine.

L'inclusion d'un gradient de concentration d'urée, orienté latéralement dans les gels de BN-PAGE, a aussi indiqué que les homodimères de NFL exprimée artificiellement chez la bactérie étaient plus stables que ceux formés par la NFL purifiée de la moelle épinière, vraisemblablement dû à des modifications post-traductionelles présentes uniquement sur cette dernière. L'interaction de ces deux types de NFL avec NFH et NFM demeurant semblables, les modifications post-traductionnelles de NFL pourraient influencer la stœchiométrie des différents dimères et permettre l'assemblage correcte de ces protéines *in vivo*. Finalement, une mutation de la NFL soupçonnée de perturber l'assemblage des NFs dans les neurones moteurs de souris transgéniques pouvait déstabiliser tout les dimères formés par cette protéine, indiquant que cette mutation perturbe le tout premier stade de l'assemblage des NFs. Le travail présenté constitue la première analyse biochimique directe de la formation et de la stabilité des dimères entre les différentes protéines de FIn.

PREFACE

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ACKNOWLEDGMENTS

The work presented in this thesis is essentially my own with the following exception. In Chapter 2, Michael G. Sacher conducted pilot experiments on the co-immunoprecipitation of the neurofilament triplet proteins from okadaic acid-treated cultures.

Chapters 2 and 3 are reprinted here with permission of the publisher and were respectively published as:

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LIST OF ABBREVIATIONS

aa	Amino acid
BisTris	Bis [2-hydroxyethyl] imino-tris[hydroxymethyl]methane
BN-PAGE	Blue native polyacrylamide gel electrophoresis
BPAG1	Bullous pemphigoid antigen 1
BPAG1n	Neuronal Bullous pemphigoid antigen 1
C-terminal	Carboxy terminal
CuP_2	Copper phenanthroline
DRG	Dorsal root ganglion
DTT	Dithiothreitol
E15	Embryonic day 15
EDTA	Ethylenediaminetetraacetic acid
GFAP	Glial fibrillary acidic protein
Glc-Nac	N-acetyl glucosamine
gua-HCl	Guanidine hydrochloride
IF	Intermediate filament
KDa	Kilodalton
KSP	Lysine-serine-proline
mAb	Monoclonal antibody
MAP	Microtubule associated protein
MF	Microfilament
MT	Microtubule
N-terminal	Amino-terminal
NF	Neurofilament
NFH	High molecular weight neurofilament subunit
NFL	Low molecular weight neurofilament subunit
NFM	Mid-sized molecular weight neurofilament subunit
nIF	Neuronal intermediate filament
OA	Okadaic acid

PBS	Phosphate-buffered saline
PKA	Protein kinase A or cAMP-dependant protein kinase
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	Tris-(hydroxymethyl)-aminomethane
Tricine	N-[2-hydroxy-1,1-bis (hydroxymethyl) ethyl] glycine
Triton	Triton X-100

CHAPTER 1

GENERAL INTRODUCTION

1– Overview Of The Neuronal Cytoskeleton

1.1– Introduction

The cytoplasm of eukaryotic cells is pervaded by a complex array of proteinaceous fibers and connecting components known as the cytoskeleton. This highly dynamic organelle provides the cell with inner structural support, anchors membrane proteins, and plays a crucial role in a variety of cell functions such as cytokinesis, cell division, and trafficking of membrane bound organelles, to name but a few. Elaboration and maintenance of the neuron's unique morphological asymmetry relies heavily upon various cytoskeletal elements to assemble growth cones, stabilize the axolemma, provide structural elements necessary for axonal transport, define and maintain axonal caliber, and provide general mechanical stability.

Three main types of protein fibers make up the cytoskeleton in neurons and in most other cells: the ~6-8 nm wide microfilaments (MFs), assembled from actin subunits, the ~10 nm wide intermediate filaments (IFs), assembled from various members of the IF family of proteins, and the ~25 nm wide microtubules (MTs), assembled from tubulin subunits. The following review will highlight the main features of microfilaments, intermediate filaments, microtubules and some of the cytoskeletal interconnecting components, and concentrate on the biology and biochemistry of neuronal intermediate filament (nIF) proteins, with a special emphasis on their assembly and related topics.

1.2– Actin Microfilaments

1.2.1– Localization Of Microfilaments In Neurons

Fluorescence staining and electron microscopy indicate that MFs are an essential component of the neuronal growth cone (Spooner and Holladay, 1981; Shaw et al., 1981) and subaxolemmal corticoskeleton (Metuzals and Tasaki, 1978; Hirokawa, 1982; Tsukita et al., 1986; Matsumoto et al.; 1989). Despite the weaker, more diffuse fluorescent staining of MFs observed in the

deeper axoplasm (Hirokawa, 1982), axoplasmic MFs actually account for >90% of the total axonal actin content in the squid giant axon (Fath and Lasek; 1988). These axoplasmic MFs are part of the previously described microtrabecular matrix (Wolosewick and Porter, 1979), a meshwork of fibers enriched in the microtubule domain which appears to link microtubules and neuronal intermediate filaments (Wolosewick and Porter, 1979; Fath and Lasek, 1988).

1.2.2– Function Of Microfilaments In Neurons

MFs are vital components of many cell functions including cytokinesis, cell anchorage, receptor organization, synaptogenesis, endocytosis (which is related to retrograde transport) and secretion (which is related to neurotransmitter release) (reviewed in Bamburg and Bernstein 1991). Movement of the leading edge of motile eukaryotic cells, which is homologous to growth cones (Small et al., 1978; Trinkaus, 1984), and general changes in cell shape in response to stimuli, are driven at least in part by MF polymerization and depolymerization cycles (Mitchison and Kirschner, 1988; Condeelis, 1993; Welnhofer et al, 1997). In recent years, mounting evidence has also accumulated pointing to a role for actin filaments in axonal transport of mitochondria, a function classically associated with microtubules (Hollenbeck, 1996).

1.2.3– Assembly Of Microfilaments

MFs are assembled from 43 KDa actin subunits. Actin is a ubiquitously expressed and highly conserved protein first purified from muscle tissue (Straub, 1942), and eventually found also in neurons (Fine and Bray, 1971). The unpolymerized "globular" and polymerized "filamentous" forms of the protein are referred to as G- and F-actin respectively. While at least six actin isoforms are expressed in mammals (Vanderchkove and Weber, 1984), the adult nervous system contains the β and γ isoforms, and several post-translational modifications such as methylation, acetylation, phosphorylation and ADP ribosylation have been reported (Bamburg and Bernstein, 1991). Incubation of

MFs with heavy meromyosin, a proteolytic fragment of myosin, leads to a unidirectional "arrowhead" decoration pattern indicating that actin filaments are polar structures (Huxley, 1963). This arrowhead pattern arbitrarily defines the "plus" (barbed) and "minus" (pointed) ends of the MF.

Assembly of G-actin into MFs is a complex process that has been extensively reviewed elsewhere (Pollard and Weeds, 1984; Frieden, 1985; Wegner, 1985; Pollard and Cooper, 1986; Lambooy and Korn, 1988; Bamburg and Bernstein, 1991, Carlier and Pantaloni, 1997). Briefly, G-actin subunits, which are normally bound to an ATP or ADP molecule, are "activated" by a conformational change that follows the binding of a Mg^{++} ion. Once activated, monomers are more likely to form dimers, a normally unfavorable reaction, sometimes followed by the formation of trimeric species. These trimers are thought to be "nuclei", operationally defined as entities which have a greater probability of elongating further than they have of breaking down into smaller species. Nuclei then elongate reversibly and bi-directionally, with the plus end growing faster than the minus end, and with ATP-actin being more likely to polymerize and not dissociate than ADP-actin. Hydrolysis of actin-bound ATP lags behind polymerization and net elongation stops when G-actin reaches the "critical concentration" where polymerization at the plus end is exactly balanced by monomer dissociation from the minus end. This creates a "treadmilling" phenomenon (Wegner, 1976) that results from the two ends of the polymer having different monomer association/dissociation rate constants.

Regulation of actin assembly can take place at many different levels. *In vitro* experiments have shown that buffer composition, especially the concentrations of Ca^{++}, Mg^{++} and KCl are very important (Frieden 1985; Pollard and Cooper, 1986). The presence of inert polymers such as dextran enhances actin polymerization in a concentration dependent manner, suggesting that the highly crowded cellular environment may also contribute to MF stability (Lindner and Ralston, 1997). As alluded to earlier, actin can undergo many post-translational modifications and the phosphorylation state of the actin-bound nucleotide moiety also influences assembly kinetics. Finally, as reviewed in the

next section, a number of MF-associated and G-actin-sequestering proteins have also been implicated in regulating the polymerization process.

1.2.4– Actin- And Microfilament-Binding Proteins

Profilin is a ubiquitous, ~15 KDa protein found initially in brain (Blickstad et al. 1980; Nishida et al, 1984a) and present in very large amounts throughout the cytoplasm (Tseng et al., 1984). It binds G-actin (Lee et al. 1982) and can prevent its polymerization (Nishida et al, 1984b). It plays a dual role in actin assembly, sequestering G-actin when the barbed ends of MFs are capped, and contributing to plus end growth upon uncapping of MFs in situations where active MF assembly is needed (Carlier and Pantaloni, 1997).

Cofilin is a member of the ubiquitous cofilin/ADF (actin depolymerizing protein) familly of actin-binding proteins. This ~19 KDa protein is found in brain (Nishida et al., 1984b), binds G-actin (Nishida et al., 1984b) and rapidly depolymerizes MFs *in vitro* (Bamburg and Bernstein, 1991) and *in vivo* (Lappalainen and Drubin, 1997). Cofilin's ability to depolymerize MFs *in vivo* was found to be essential for endocytosis in yeast mutants (Lappalainen and Drubin, 1997). Moreover, a recent model of the "funneled treadmilling process" (Carlier and Pantaloni; 1997) proposes that actin depolymerizing proteins such as cofilin or ADF act in concert with barbed end capping proteins to rapidly dissassemble a large number of MFs from the minus end. The resulting increase in the steady-state amount of G-actin would promote very rapid growth of a few uncapped filaments, an important factor in actin-based filopodial extension. Other agonists of actin filament turnover are also expected to take part in this process to generate some of the polymerization velocities observed *in vivo* (Carlier and Pantaloni, 1997).

Myosins constitute a very large family of related actin-based motor proteins, and as many as thirteen classes have been identified (Cope et al., 1996). In neurons, myosin V is believed to be a strong candidate as a motor for actin-based, membranous organelle transport (Goodson et al., 1997).

Other important actin-binding proteins include synapsin 1, a major neuronal phosphoprotein (De Camilli and Greengard, 1986), fodrin (brain spectrin, calspectin), and tropomyosin, better known for its role in muscle contraction. Synapsin 1 appears to cross-link synaptic vesicles to one another (Landis et al. 1988; Hirokawa et al. 1989) and to the three types of cytoskeletal filaments (reviewed in Hirokawa, 1991 and Cheek and Burgoyne, 1991). Fodrin is related to spectrin, a large (10^6 KDa), heterotetrameric erythrocyte protein that connects filamentous actin to the plasma membrane, making it an important component of the cortical cytoskeleton (reviewed in Baines, 1991). Fodrin was recently shown to also bind synapsin 1 (Iga et al., 1997). Interestingly, Ca^{++}-induced phosphorylation of synapsin 1 both regulates neurotransmitter release (Cheek and Burgoyne, 1991; Hicks et al., 1997), and inhibits its binding to fodrin, suggesting that fodrin may be involved in the former phenomenon (Iga et al. 1997). Tropomyosin is a dimer of 35KDa subunits that binds along actin filaments, inhibits actin interactions with myosin in muscle (Huxley, 1969), and stabilizes MFs in many cell types (Bamburg and Bernstein, 1991). Although the precise function of tropomyosin in neurons is still unclear, different isoforms have been found to associate with different types of MFs located in neurites or growth cones (Schevzov et al. 1997).

1.3– Tubulin And Microtubules

1.3.1– Localization Of Microtubules In Neurons

In immunofluorescence studies of cultured neurons, microtubules (MTs) were found in all neuronal compartments, albeit at lower levels in the growth cone (Spooner and Holladay, 1981). Freeze-etch electron microscopy observation of large, myelinated axons revealed that MTs form small longitudinal bundles interspersed throughout the axoplasm, and that numerous side arms appear to connect them to one another, to neurofilaments, and to membrane-bound organelles (Hirokawa 1982, 1991). Biochemical studies have indicated that MTs are abundant both in the cortical

region and the central part of axoplasm (Matsumoto et al., 1989). In neurons, decoration studies with motor molecules have shown that the majority of axonal MTs are oriented with their plus end (see later) distal to the perikaryon (Burton and Paige, 1981; Heidemann et al., 1981).

1.3.2– Function Of Microtubules In Neurons

One of the better characterized function of MTs in neurons concerns their role as "tracks" supporting both retrograde and anterograde "fast" axonal transport of membrane-bound organelles (reviewed in Bloom et al., 1989; Allan et al., 1991; Goodson et al., 1997). This ATP-dependent (Adams, 1982; Brady et al., 1982) form of transport persists for several hours in axoplasm extruded from the squid giant axon (Brady et al., 1982), and microtubules were found to support movement in both directions in that system (Schnapp et al., 1985). More recent evidence indicates that MTs may also support the "slow" type of axonal transport used for neurofilament proteins (Terada et al., 1996, and see section on axonal transport).

In addition, a role for MTs in axonal growth has also been reported. Indeed, topical application of MT depolymerizing drugs at the axon tip was shown to slow down axonal growth (Yu and Baas, 1995; Rochlin et al., 1996) and compromise the growth cone's ability to find its proper path (Tanaka et al., 1995).

1.3.3– Structure And Assembly Of Microtubules

Microtubules are assembled from tubulin, a heterodimer (Ludueña et al., 1977) consisting of ~50 KDa "α" and "β" polypeptides that bear ~60% sequence homology (Ponstingl et al., 1981; Krauhs et al., 1981; Valenzuela et al., 1981). The tubulin heterodimer is assembled in a head to tail fashion, thereby creating a polarity in MT structure, with a fast growing, β-tubulin-bound, "plus" end, and a slow growing, α-tubulin-bound, "minus" end (reviewed in Mandelkow and Mandelkow, 1989, and Wade and Hyman, 1997). Simlar to what occurs with MFs, the different assembly kinetics at the ends of the

filament result in treadmilling (Margolis and Wilson, 1978) and fluctuations in individual MT lengths referred to as "dynamic instability" (Mitchison and Kirschner, 1984).

Electron microscopy observation of assembled MTs shows them to be tubular structures resulting from lateral associations of 9 to 16 linear protofilaments (reviewed in Linck, 1989). However, recent examination of growing MTs indicates that tubulin dimers assemble on two-dimensional sheets that continuously "zip up" into the tubular structure normally observed (Chrétien et al. 1995).

Although nucleation in MT assembly can occur spontaneously *in vitro*, *in vivo* assembly normally proceeds from microtubule organizing centers on centrosomes (Osborn et al., 1976; Frankel, 1976), where γ-tubulin (Oakely and Oakely, 1989; Oakely, 1992) is organized in a ring structure that nucleates MT assembly at the minus end, and dictates the 13-protofilament structure most commonly observed *in vivo* (Moritz et al., 1995; Zheng et al., 1995). The precise way in which nucleated rings proceed to (plus end-directed) sheet-like growth has yet to be elucidated (Wade and Hyman, 1997). Interestingly, katanin, an ATP-dependent MT-severing protein, has been located near centrosomes (McNally et al., 1996), where it could potentially release assembled MTs for transport down the axon (Baas, 1997). Intriguingly, the disassembly of MTs was shown to proceed through a very different mechanism involving peeling and curling of individual protofilaments into tight rings (Mandelkow and Mandelkow, 1989; Mandelkow et al. 1991).

Both α- and β-tubulin have a bound GTP moiety (Jacobs et al., 1974), but only GTP bound to β-tubulin is exchangeable (Geahlen and Haley, 1977; Zeeberg and Caplow, 1979), and is normally hydrolyzed following polymerization (Jacobs et al., 1974; Weisenberg et al., 1976; Zeeberg et al., 1980). The role of GTP hydrolysis in assembly remains unclear, although the fact that MTs grown in the presence of a slowly-hydrolyzing or non-hydrolyzable GTP analog are much more stable than GDP-MTs indicates that it is not

essential for assembly, and further suggests that it probably plays a role in regulating the dynamic instability of MTs (Hyman et al., 1995).

A number of post-translational modifications of tubulin have been reported. For instance, *in vivo* labeling indicated that α-tubulin can be acetylated on a lysine ε-amino group (L'Hernault and Rosenbaum, 1985), later identified as amino acid residue 40 (LeDizet and Piperno, 1987). Although acetylated tubulin is often associated with more stable MTs, its absence in certain cells indicates that it is not essential for survival. Moreover, transfection experiments where a non-acetylatable mutant accounted for 70% of the tubulin present failed to reveal any obvious phenotype (MacRae, 1997). α-Tubulin may also undergo a reversible loss of the C-terminal tyrosine residue found in most isoforms (Barra et al., 1973, 1974; Hallak et al., 1977, Greer and Rosenbaum, 1989). Detyrosination of tubulin appears to be a consequence, rather than a cause, of its association with stable MTs, which are the preferred substrate for tubulin carboxypeptidase (Wehland and Weber, 1987). Tyrosine-ligase preferentially adds tyrosine to the C-terminus of α-tubulin in soluble tubulin dimers, and knowledge of its sequence (Ersfeld et al., 1993) may help shed more light on the function of tubulin tyrosination-detyrosination (MacRae, 1997). Since the discovery of β-tubulin phosphorylation by Eipper (1974), numerous kinases, including tyrosine kinases, have been implicated as possible α- or β-tubulin kinases (MacRae, 1997). Although phosphorylation of tubulin is often associated with differentiation and with more stable MTs in neurons, its function remains unclear at present (Greer and Rosenbaum, 1989; MacRae, 1997). Polyglutamylation and polyglycylation of both tubulin subunits have been reviewed elsewhere, and their function is still undetermined (MacRae, 1997).

1.3.4.– Microtubule-Binding Proteins

MTs are commonly purified by polymerization-depolymerization cycles coupled with centrifugation steps to eliminate contaminants. Two proteins with molecular masses >250 KDa, microtubule associated proteins 1 and 2 (MAP 1

and MAP2), were found to co-purify with MTs (Borisy et al, 1975; Sloboda et al., 1975; Dentler et al., 1975), and were later resolved into five proteins, MAPs 1A, 1B, 1C and MAPs 2A and 2B (Bloom et al., 1984a, b).

MAP 1C was found to be a cytoplasmic equivalent of dynein, and is thought to be the motor molecule responsible for retrograde transport in neurons (Paschal et al., 1987a, b).

Antibody decoration showed that MAPs 1A and 1B form the side arm projections that appear to connect MTs in axons and dendrites, while MAP 2 showed a similar profile in dendrites only (Hirokawa et al., 1985; Shiomura and Hirokawa, 1987; Hirokawa et al., 1988a). It is now clear that MAPs also control MT dynamics *in vitro* as well as *in vivo* (Hirokawa et al., 1988a; Hirokawa, 1994). In this latter respect, anti-sense oligonucleotides to the mRNA for MAP 2C, a juvenile form of MAP 2 (Garner and Matus, 1988), inhibited the initial phase of neurite outgrowth in cultured neurons (Caceres et al., 1992).

The tau family of proteins, with a molecular mass range of 55-62 KDa, co-purifies with tubulin and promotes MT assembly *in vitro* (Cleveland et al., 1977a, b). Tau is abundant in axons where it localizes close to MTs in immunofluorescence studies (Binder et al., 1985). Tau also forms cross-bridges between MTs in vitro (Hirokawa et al., 1988b), and transfection of its cDNA into fibroblasts promotes MT bundling in a pattern reminiscent of that seen in axons (Kanai et al., 1989). Treatment of cultured neurons with anti-sense oligonucleotides further revealed that initiation and elongation of neurites required the participation of tau (Caceres and Kosik, 1990; Shea et al., 1992).

In some transfection experiments MAP 2C (Edson et al., 1993) and tau (Baas et al., 1991) can induce process formation in non-neuronal cells. Considering also that transfection of non-neuronal cells with MAP 2, MAP 2C and tau can induce MT bundling with spacings similar to those observed in their *in vivo* neuronal location (Chen et al., 1992), it appears that these proteins play an important role in neuronal morphogenesis (Hirokawa, 1994).

1.4– The Intermediate Filament Family Of Proteins

1.4.1– Localization Of Intermediate Filament Proteins

IFs are assembled from various members of a large family of related proteins which are mostly expressed in a tissue-specific manner (Steinert and Roop, 1988; Fuchs and Weber, 1994). As summarized in Table 1, IF proteins are subdivided into six groups based on sequence homology and intron/exon organization. Types I and II are acidic and basic keratin subunits respectively and are found in epithelial cells (Steinert and Roop, 1988). Type III includes desmin, found in muscle cells, glial fibrillary acidic protein (GFAP), found in glial cells (Steinert and Roop, 1988) and peripherin, found in neurons with peripheral projections (Portier et al., 1984; Parysek et al., 1991). Type IV includes the neurofilament (NF) triplet protein NFH, NFM and NFL (see later) (Steinert and Roop, 1988) as well as α-internexin (Fliegner et al., 1990). Type V includes the lamins, found in all cell nuclei (Steinert and Roop, 1988) while type VI includes nestin, an IF protein found in neuron and muscle precursor cells (Lendhal et al., 1990; Zimmerman et al., 1994).

IFs are often concentrated in the perinuclear region, where they appear to be anchored to and perhaps position the nucleus (Skalli and Goldman, 1991). From there, they extend throughout the cytoplasm, and attach to desmosomes and hemidesmosomes at the plasma membrane through the protein desmoplakin, as was shown by transfection experiments with vimentin- and cytokeratin-containing cells (Green and Jones, 1990, 1996; Stappenbeck et al., 1993; Bornslaeger et al., 1996).

In mature neurons, and especially in large myelinated axons, NFs constitute the major cytoskeletal component, although they are more sparse towards the periphery of axons and are present in only small amounts in the dendrites of most neurons (Hirokawa, 1991). NFs are assembled from the low (NFL, 62 KDa, Chin and Liem, 1989), mid-sized (NFM, 95 KDa, Napolitano et al., 1987) and high (NFH, 115 KDa, Chin and Liem, 1990) molecular weight subunits (The respective molecular masses indicated in parentheses pertain

Type	IF Protein	Molecular Mass	Localization
I	Acidic Keratins[1]	~40-60 KDa	Epithelial Cells
II	Basic Keratins[1]	~50-70 KDa	Epithelial Cells
III	Desmin[1]	~ 52 KDa	Muscle Cells
	GFAP[1]	~ 51 KDa	Glial Cells
	Vimentin[1]	~ 53 KDa	Cells Of Mesenchymal Lineage, Many Cultured Cells, Embryonic Neurons
	Peripherin[2]	~ 55 KDa	Peripheral Neurons
IV	NFH[3]	~ 115 KDa	Most Neurons
	NFM[4]	~ 95 KDa	Most Neurons
	NFL[5]	~ 62 KDa	Most Neurons
	α-Internexin[6]	~ 66 KDa	Most Neurons
V	Lamins[1]	~ 60-70 KDa	All Cell Nuclei
VI	Nestin[7]	~ 200 KDa	Neuron And Muscle Precursor Cells

[1] Steinert and Roop, 1988
[2] Leonard et al., 1988, Parysek et al., 1988
[3] Chin and Liem, 1990
[4] Napolitano et al., 1987
[5] Chin and Liem, 1989
[6] Fliegner et al., 1990
[7] Lendhal et al., 1990

Table 1: List of mammalian intermediate filament proteins classified according to their type and indicating their approximate deduced molecular mass and usual tissue distribution. Some of the apparent molecular masses on SDS gels may differ. See text for additional references.

to the rat NF subunits). In addition, peripheral and central axons may contain variable amounts of peripherin (Portier et al., 1984; Parysek et al., 1991) and/or α-internexin (Chiu et al., 1989, Kaplan et al., 1990), respectively.

1.4.2– Expression Of Neuronal Intermediate Filament Proteins During Development

Neurons express a variety of IF proteins in a developmentally and spatially regulated fashion. As neurons differentiate, expression of vimentin (Tapscott et al., 1981; Bignami et al., 1982; Cochard and Paulin, 1984) and nestin (Lendhal et al., 1990) is replaced with that of other IF proteins. In central neurons, α-internexin expression coincides with neuronal differentiation, usually preceding that of NFM and NFL, and declining sharply in the adult (Kaplan et al., 1990; Fliegener et al., 1994). NFH expression follows those of NFL and NFM, later in development, usually after synapse formation and during myelination (Shaw and Weber, 1982; Pachter and Liem, 1984; Carden et al., 1987). Peripherin is restricted to neurons of neural crest origin or to neurons that project to the periphery (Parysek and Goldman, 1988; Escurat et al., 1990; Gorham et al., 1990; Troy et al., 1990a) and its expression follows that of NFL in development (Escurat et al., 1990, Gorham et al., 1990). In the adult, peripherin expression drops sharply in large myelinated motor neurons, remains higher than NF expression in smaller, non-myelinated sensory neurons, and is similar to NF protein expression in medium-sized neurons (Escurat et al., 1990; Gorham et al., 1990; Troy et al., 1990a; Goldstein et al., 1991). Interestingly, the expression profile of nIF protein during neuronal regeneration tends to recapitulate that observed in development, and the upregulation of peripherin in regenerating myelinating axons suggest that it may play a role in this situation (Oblinger et al., 1989).

1.5– Integration Of The Three Cytoskeletal Components

Although it is practical to discuss MFs, MTs and IFs separately, these three components overlap extensively in the cell (Hirokawa, 1991), and their

structural integrity is often interdependent. For instance, microinjection of fibroblasts with peptides that cause vimentin disassembly also leads to a complete collapse of the MT and MF networks (Goldman et al. 1996). A growing number of molecules are coming under scrutiny for their possible role as cross-linking elements between the three major cytoskeletal fibers. In this regard, bullous pemphigoid antigen 1n (BPAG1n), a neuronal relative of BPAG1 (Brown et al., 1995), was shown to have a coiled-coil rod domain flanked by a NF and a MF binding site that mediate cross-linking of these two arrays both *in vitro* and *in vivo* (Yang et al., 1996).

Plectin, a protein related to BPAG1, has been shown to bind fodrin, MAPs, and various types of IF proteins *in vitro* (Green and Jones, 1996). Immunogold labeling of cultured fibroblasts further showed that plectin side arms connect vimentin to microtubules directly, and to actin stress fibers through myosin II (Svitikana et al. 1996).

Several microtubule binding proteins have also been found to interact with other cytoskeletal elements. For instance, *in vitro* experiments showed that MAP 2 and tau bind to actin (Kotani et al., 1985; Sattilaro, 1986) and to NFL (Heimann et al., 1985; Miyata et al., 1986). Furthermore, process formation was found to be crucially dependent upon MAP 2C interactions with both MTs and MFs *in vivo* (Cunningham et al., 1997).

2– Structure Of Neuronal Intermediate Filament Proteins

2.1– Overall Domain Organization

As depicted in Fig.1, the sequence of IF proteins can be divided into three distinct domains. The N-terminal "head" and C-terminal "tail" domains vary in length and composition while the central "rod" domain is relatively well conserved among different IF proteins. Homologies in amino acid sequence and intron organization of the rod domain permit the IF protein classification

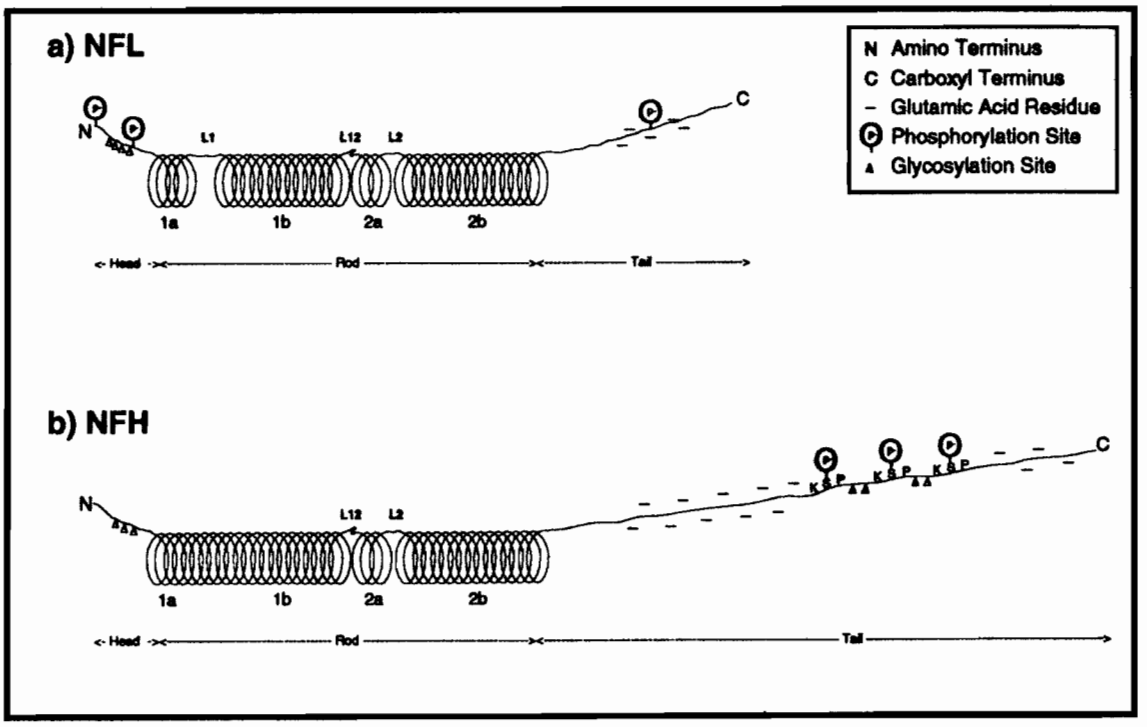

Figure 1: Diagrammatic representation of NFL and NFH. a) NFL. The α-helical, heptad bearing coils 1a, 1b, 2a and 2b are separated by the L1, L12 and L2 linker regions. The approximate locations of the 4 glycosylation sites (Threonine 21, Serines 27, 34 and 48) and the three phosphorylation sites (Serines 2 and 55 in the head domain and Serine 473 in the tail domain) are indicated. b) NFH. Note the continuous coil 1 segment. For simplicity, only 3 of the 52 KSP repeats in rat NFH and only 4 of the 8 glycosylation sites in the tail domain are indicated. The head domain also contains multiple glycosylation sites (Threonine 53, Serines 54 and 56). References are cited in the main text.

scheme presented in Table 1 (Steinert and Roop, 1988; Fuchs and Weber, 1994).

2.2– The N-Terminal Head Domain

2.2.1– Overall Structure

The head domain in nIF proteins is ~100 amino acid (aa) long and is believed to contain a number of β-type motifs (Napolitano et al., 1987; Chin and Liem, 1989, 1990; Shaw, 1991; Parysek et al., 1988; Leonard et al., 1988; Fliegner et al., 1990). Moreover, this domain is typically rich in serine and threonine residues involved in phosphorylation and glycosylation, which are potential regulators of assembly (see below). The head domain of α-internexin bears about 33% aa sequence homology with NFM (Fliegner et al., 1990). The head domain of peripherin is most homologous to the NFL head domain (Leonard et al., 1988), a peculiar feature considering that peripherin is a type III IF protein. There is no evidence yet regarding possible glycosylation of α-internexin or peripherin.

2.2.2–The Head Domain Is Exposed On The Surface Of Filaments

Immunoelectron microscopy using the few monoclonal antibodies (mAbs) that have been obtained against the head domain of NF proteins reveals that it is at least partially exposed on the surface of the filaments (Balin et al., 1991; Balin and Lee, 1991).

2.3– The α-Helical Rod Domain

2.3.1– Overall Structure

The rod domain is ~310 aa in length for mammalian cytoplasmic IF proteins, and is characterized by the presence of four stretches of aa heptad

repeats of the form $(abcdefg)_n$, where hydrophobic aa residues occupying the "a" and "d" positions favor the formation of a coiled-coil dimer (Geisler and Weber, 1982; Geisler et al., 1983; 1984; 1985b). These stretches of heptad repeats are referred to as the 1a, 1b, 2a, and 2b coiled-coil segments and are separated by the short "linker" regions L1, L12, and L2 which do not contain the heptad motif (Fig.1). Interestingly, whereas most IF proteins, including NFL, α-internexin and peripherin, bear a helix-disrupting proline residue in their L1, non α-helical linker, NFH and NFM do not, suggesting that their coil 1 is uninterrupted (Lees et al., 1988; Myers et al., 1987; Schneidman et al., 1988). The short L12 linker may adopt a β-conformation (Steinert and Roop, 1988), while the L2 linker is α-helical but does not conform to the heptad repeat pattern (Shaw, 1991). Coil 2b contains a conserved tryptophan residue whose insertion causes a reversal in the heptad repeat pattern, although its significance remains unknown (Shaw, 1991). Maximum homology between IF proteins can be found both at the beginning of coil 1a, and at the end of coil 2b where a highly conserved consensus sequence constitutes an epitope common to all IF proteins (Pruss et al., 1981; Fuchs and Weber, 1994). As will be seen later, these conserved areas play an important role in IF assembly.

2.3.2– The Rod Domain Forms The Filament Core

As alluded to earlier, the aa sequence of the rod domain can direct coiled-coil dimer formation, the essential first step in IF assembly. Moreover, the small size of the head domain, and the fact that in some non-neuronal IF proteins, the tail domain is dispensable for assembly (reviewed in Fuchs and Weber, 1994) strongly suggest that the rod domain forms the core of assembled IFs. The inability of rod domain-directed mAbs to bind to native or reassembled, heteropolymeric NFs, was taken to mean that this domain is buried within the core of the filament where the relevant epitopes are masked (Balin et al., 1991). This was corroborated by the fact that the core of *in vitro*

reassembled homopolymeric NFs could in fact be labelled with these rod domain-specific mAbs, presumably because different packing constraints in these filaments allowed exposure of their epitopes (Balin and Lee, 1991; Mulligan et al., 1991). In a more recent study, rod-directed mAbs were shown to label the core of native NFs in axons that had been damaged *in vivo* by impact injury. In this system, the damage resulted in a loss of NFH and NFM tail piece extensions in the injured area which apparently allowed the mAbs to access the rod domain epitopes (Povlishock et al., 1997).

2.4– The C-Terminal Tail Domain

2.4.1– Overall Structure Of The Tail Domain

Differences in length of the tail domain of NFL (~140 aa, Chin and Liem, 1989), NFM (~438 aa, Napolitano et al., 1987), and NFH (~664 aa, Chin and Liem, 1990) account for differences in the subunit molecular masses. These domains are rich in glutamic acid residues, and in the case of rat NFM and NFH, they respectively contain 5 and 52 lysine-serine-proline (KSP) repeats (Napolitano et al., 1987; Chin and Liem, 1990). The C-terminus of NFM is also unusual in that it contains short sequences that are virtually identical among species as different as chicken and human (Myers et al, 1987; Shaw, 1989). The low mAb staining of these seemingly accessible epitopes suggests that they are masked, perhaps due to assembly-related interactions (Balin and Lee, 1991), although more rigorous proof of this is still lacking.

Although the rod domain of α-internexin most resembles that of NFL because it contains a very similar L1 linker region, its tail domain contains stretches resembling the tail domain of NFM, giving α-internexin a hybrid character (Fliegner et al., 1990; Lee and Cleveland, 1996). On the other hand, the tail domain of peripherin most resembles that of desmin and vimentin, consistent with these proteins being part of the type III IF protein subfamily (Leonard et al., 1988).

2.4.2– The Tail Domains Of NFH And NFM Form Cross-Bridges Or Spacer Arms

Examination of freeze-fractured axoplasm by electron microscopy typically reveals the presence of cross-bridges that appear to cross-link NFs to each other or to axonal structures such as MTs or other organelles (Hirokawa, 1982). Lacking in other IFs, these cross-bridges are typically 30 to 50 nm in length, 4 to 6 nm-thick and 25 to 100 nm apart (Hirokawa et al., 1984; Ip et al., 1985b; Eagles et al., 1990; Hirokawa, 1991; Gotow et al., 1992). Several lines of evidence indicate that NF cross-bridges are formed by the tail domains of NFH and NFM. Early immunoelectron microscopy studies revealed that antibodies directed against NFH labelled cross-bridges *in situ* (Willard and Simon, 1981; Sharp et al., 1982; Hirokawa et al., 1984). Subsequently, *in vitro* reconstituted NFs containing NFL alone were shown to have the smooth surface typical of other IFs, while filaments reconstituted from NFL and NFM or NFL and NFH showed projections with a ~22 nm spacing that were ~4 nm-thick and had average lengths of 55 nm and 63 nm, respectively, consistent with the size difference between the tail domains of NFM and NFH (Hisanaga and Hirokawa, 1988). A similar study corroborated these results and suggested that NFM projections do not necessarily connect with other structures while NFH projections are more likely to do so (Gottow et al., 1992). Furthermore, a detailed immunoelectron microscopy study using mAbs against well-defined epitopes clearly demonstrated that the tail domains of NFH and NFM were responsible for the formation of cross-bridges in both native and reconstituted NFs (Mulligan et al., 1991). Finally, co-transfection of NFL and NFM in IF$^-$ Sf9 insect cells also indicated that the tail domain of NFM was a structural component of NF cross-bridges (Nakagawa et al., 1995).

Although ultrastructural studies clearly show contact sites between the tail-piece extensions of NFH and NFM and other structures, current evidence suggests that they may not constitute true cross-bridges but rather may act as spacer arms. For instance, altough extensively dephosphorylated NFH does

appear to interact with assembled MTs *in vitro*, this NFH isoform is normally rare in the axonal compartment (Hisanaga and Hirokawa, 1990a). Meanwhile, purified tail domains from NFH and NFM were shown not to interact significantly with native NFs, regardless of their phosphorylation state (Chin et al., 1989). Furthermore, NFs and MTs from extruded squid axoplasm were shown to continuously disperse radially once the constraining presence of the plasma membrane was removed, indicating that they were inherently non-interactive (Brown and Lasek, 1988).

Although the NFH and NFM tail domains may not actively cross-link structures in the axoplasm, their presence and size does seem to specify, at least to some degree, the interfilament spacing *in vivo*. Indeed, the interfilament spacing varied according to the size of various tail deletion mutants of NFM co-transfected with NFL in IF⁻ Sf9 cells (Nakagawa et al., 1995). Moreover, impact acceleration injury induces NF compaction in rats, concomitant with the loss of their NF tail piece extensions (Pettus and Povlishock, 1996; Povlishock et al., 1997).

2.5– Post-Translational Modifications Of Neuronal Intermediate Filament Proteins

2.5.1– Phosphorylation

One of the most conspicuous features of NF proteins is their high phosphate content. Rat NFL, NFM and NFH were shown to contain 3, 6, and 14 moles of phosphate per mole of polypeptide (Julien and Mushynski, 1982). These values are lower than the ones initially reported, and take into account the subsequently determined true molecular masses of the subunits. Indeed, rat NFL, NFM and NFH migrate on SDS-PAGE with apparent molecular masses of ~70 KDa, ~150 KDa, and ~200 KDa (Hoffman and Lasek, 1975), respectively. These values differ substantially from the actual molecular masses estimated from sequence data (~62 KDa, ~95 KDa, and 115 KDa, respectively). The anomalous gel electrophoretic mobility has been attributed to

the high phosphate and glutamate content of the subunits (Julien and Mushynski, 1982, Kaufman et al., 1984; Georges and Mushynski, 1987). Proteolytic cleavage of NFM and NFH further indicated that the bulk of phosphorylated serine residues was concentrated in the tail domain (Julien and Mushynski, 1983).

A more detailed study of proteolytic fragments from the tail domain identified the KSP regions of porcine NFM and NFH as the main phosphorylation sites in these proteins (Geisler et al., 1987). This was confirmed using a different approach where a collection of mAbs to native and variously dephosphorylated forms of NFH and NFM was tested against various synthetic peptides (Lee et al., 1988). More recently, the tail domain of NFH was extensively analyzed by a combination of proteolytic and chemical cleavage, protein sequencing and mass spectrometry. This study confirmed that phoshorylation of this domain takes place mostly on the 52 KSP repeats present there, and a cyanogen bromide fragment containing 43 KSP repeats was shown to contain 30 to 35 mol of Pi/mol of protein (Elhanany et al., 1994). A single phosphorylation site consisting of Serine 473 has been identified in the tail domain of NFL (Xu et al., 1990).

Head domain phosphorylation has been documented for NFM (Sihag and Nixon, 1990), NFL (Sihag and Nixon, 1989; Sacher et al., 1994; Giasson et al., 1996), α-internexin (Tanaka et al., 1993) and peripherin (Huc et al., 1989) and is associated with the regulation of filament assembly (Nixon and Sihag, 1991).

2.5.2–Phosphorylation Of NF Proteins Is Spatially Regulated

Numerous immunohistochemical studies using mAbs specific for various NF protein phosphovariants have indicated that NF proteins, especially NFM and NFH, are more heavily phosphorylated in the axon than in dendrites or perikarya, and that these differences involve tail domain phosphorylation (Sternberger and Sternberger, 1983; Lee et al., 1987; 1988). Moreover, the hyperphosphorylated forms of NFH turn over very slowly in the axonal

compartment (Lewis and Nixon, 1988; Nixon et al., 1994a). Interestingly, phosphate moieties present on the head domain of NFL and NFM at the time of synthesis in the perykaryon are either lost or turnover very rapidly as the proteins are transported down the proximal axon (Sihag and Nixon, 1989; 1990; Nixon, 1991).

2.5.3– Possible Roles Of Tail Domain Phosphorylation

The large number of phosphate moieties in NFM and especially in NFH, raises obvious questions regarding their function. Phosphorylated NF proteins have been found to be more resistant to proteolysis than their hypophosphorylated counterparts, suggesting that phosphorylation may protect these proteins from degradation during their typically long residence time in the axon (Goldstein et al., 1987; Pant, 1988; Elhanany et al., 1994). The fact that hyperphosphorylated forms of NFH are associated with a more static pool of axonal NFs (see below) also suggests that phosphorylation may regulate axonal transport of NF proteins (Lewis and Nixon, 1988).

Given that NFs appear to be intrinsic determinants of axonal caliber (Hoffman et al., 1984; 1985; 1987; Yamasaki et al., 1991; 1992; Eyer and Peterson, 1994; Zhu et al., 1997), a frequently quoted hypothesis proposes that tail domain phosphorylation creates repulsive forces that result in hyperextension of these domains with a concomitant increase in interfilament spacing and axonal caliber (Carden et al., 1987). In fact, electron microscopy reveals that axonal, hyperphosphorylated NFs, have a greater interfilament spacing than dendritic, hypophosphorylated filaments (Hirokawa, 1984). Similarly, transplanting myelin-competent Schwann cells into myelin-deficient trembler mice results in localized, myelin-induced increases in axonal diameter that are correlated with increases in NFH phosphorylation (deWaegh et al., 1992). This effect of myelination has subsequently been documented in several laboratories (Hsieh et al., 1994b; Cole et al., 1994; Nixon et al., 1994b). In addition, the nodes of Ranvier of myelinated axons show both a markedly reduced axonal caliber, and lower levels of NF phoshphorylation (Mata et al.,

1992; Hsieh et al., 1994b). Furthermore, transgenic mice overexpressing the human NFM subunit showed increased packing NF density and a reduction in hyperphosphorylated NFH (Tu et al., 1995).

This type of correlative work should, however, be interpreted cautiously. For instance, enzymatic dephosphorylation of NFs did not alter the length of their sidearms when viewed by rotary shadowing electron microscopy (Hisanaga and Hirokawa, 1989). Moreover, transgenic mice overexpressing the murine NFM subunit showed reduced levels of the NFH protein and reduced axonal caliber, but no significant changes in interfilament spacing (Wong et al., 1995). Finally, a more recent study involving normal and regenerating lamprey axons indicated that small axons could be found with loosely packed, hypophosphorylated NFs. At the same time, tightly packed NFs were found that were highly phosphorylated, prompting the authors to conclude that "...phosphorylation of NFs does not rigidly determine their spacing and NF spacing does not rigidly determine axon caliber." (Pijak et al., 1996).

2.5.4– Glycosylation Of Neurofilament Proteins

A growing number of phosphoproteins are being shown to also undergo *O*-linked glycosylation. The linkage of N-acetylglucosamine (*O*-GlcNac) to serine or threonine residues appears to be both widespread and as dynamic as phosphorylation (Haltiwanger et al., 1992). Interestingly, all three NF subunits have been shown to contain 3 to 4 *O*-GlcNac sites in their head domains, and at least 8 additional sites were mapped to the KSP region of the NFH tail domain (Dong et al., 1993; 1996). The stoichiometry of glycosylation varied from 0.1 to 0.3 moles of GlcNac per mole of polypeptide, suggesting that the individual sites are only sporadically modified. However, the authors point out that these values are averaged over the entire NF population, and that a subset of subunits could contain the bulk of glycosylated residues with a much higher stoichiometry. Although the role of NF protein glycosylation remains to be ascertained, the modifications occur in regions that are also subject to phosphorylation, and whose deletion markedly affects subunit assembly in

transfected cells. This points to a possible regulatory role for *O*-linked glycosylation in NF assembly (Dong et al., 1993, 1996). Dynamic glycosylation has also been observed in the keratins (Chou et al., 1992), suggesting that it may represent a common form of IF protein modification.

3– Intermediate Filament Assembly

3.1– Intermediate Filaments Readily Form *In Vitro*

IF proteins denatured by urea or guanidine share two essential characteristics. Firstly, they readily self-assemble *in vitro* into normal-appearing filaments upon dialysis against near-physiological buffers. Secondly, small dimeric or tetrameric soluble species are readily generated by using appropriate urea or guanidine concentrations, or by the use of low ionic strength buffers at alkaline pH (e.g., see Quinlan et al., 1984, 1986; Carden and Eagles, 1983; Cohlberg et al., 1987; Hisanaga and Hirokawa, 1990b; Hisanaga et al., 1990b; Geisler et al., 1992; Steinert et al., 1993 and other references covering this section). These and other features described hereafter have helped elucidate several aspects of IF assembly.

3.2– α-Helices And Coiled-Coil Interaction The α-helix is a widely recurring motif of protein secondary structure where the α-carbonyl group of every aa at position "i" forms a hydrogen bond with the α-amide group of the aa at position "i+4" to give the protein backbone a helical structure containing ~3.6 residues per 360° turn (Pauling and Corey, 1953). This helical structure can be further stabilized in solution by intrahelical salt bridges between oppositely charged side-chains of aas at positions "i" and "i+4" (Fig.2) (reviewed in Letai and Fuchs, 1995).

An important class of α-helices contains heptad repeats of the form $(abcdefg)_n$, where positions "a" and "d" are occupied primarily by hydrophobic aa residues (Crick, 1953). When a peptide contains a minimum of ~4 heptads

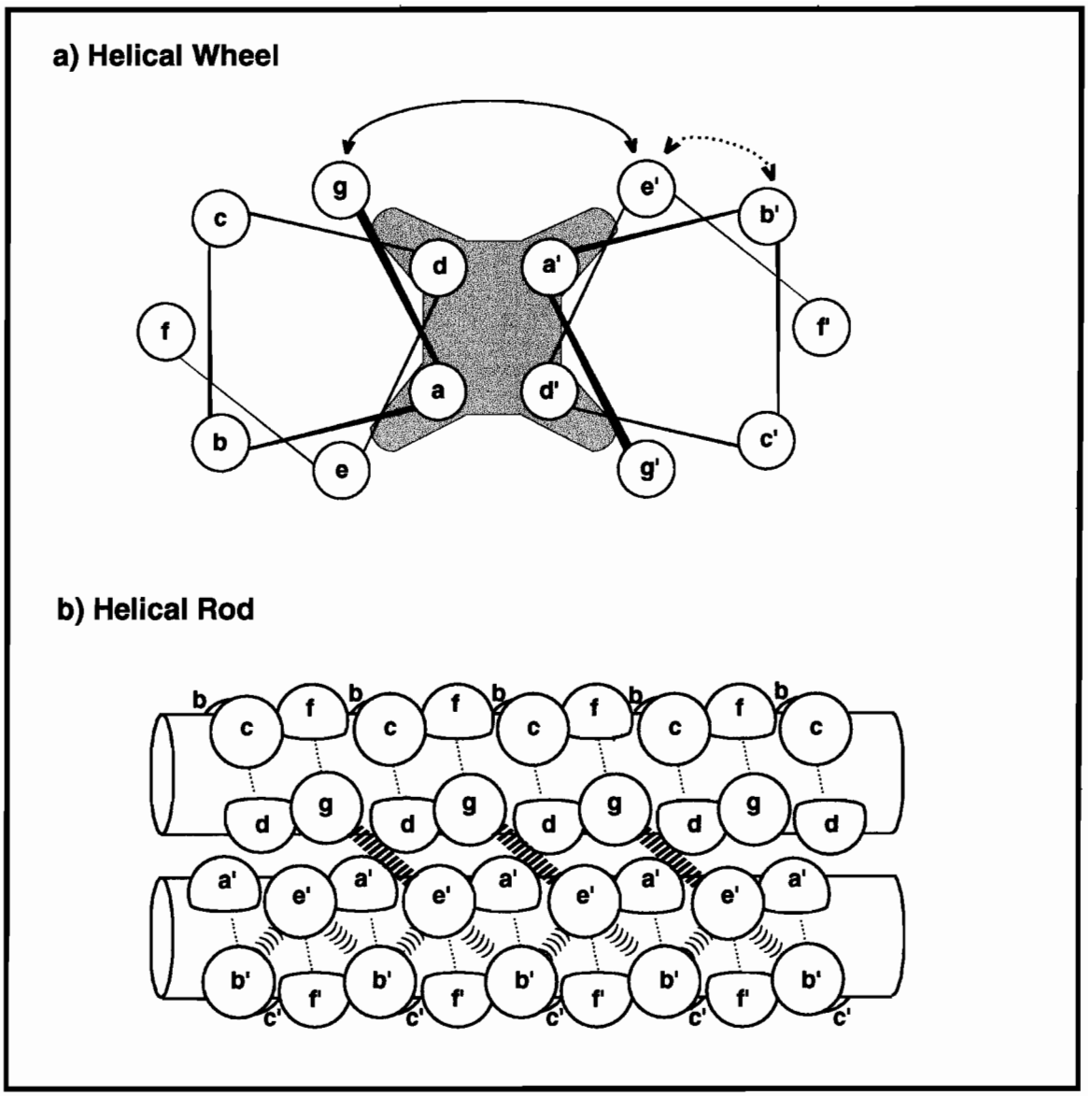

Figure 2: Diagrammatic representation of α-helices arranged in a coiled-coil dimer. a) Helical wheel representation. The amino-acids making up a heptad repeat are labelled a-g for one α-helix and a'-g' for the other α-helix. The shaded area represents the region involved in hydrophobic interactions. The solid and doted arrows represent interhelical and intrahelical salt bridges, respectivley. b) Helical rod representation. The amino-acids are labelled as for figure 2a. Interhelical and intrahelical salt bridges are represented by "*////////*" and "))))))" respectively. References are cited in the main text.

(Lau et al., 1984, Su et al., 1994, Lumb et al., 1994), two or more such species can intertwine to form a coiled-coil structure where residues "a" and "d", and to a lesser extent, the methylene groups of residues "e" and "g", form a stabilizing hydrophobic seam (reviewed in Lumb and Kim, 1995). Packing constraints dictate that the presence of isoleucine and leucine at positions "a" and "d", respectively, favors dimerization, while other arrangements can promote trimeric or tetrameric associations, underscoring the major importance of these residues for coiled-coil interaction (reviewed in Nautyal et al., 1995). As shown in Fig.2b, the charged residues at positions "e" and "g" are strategically located to also influence the pairing specificity of the helices. For instance, repulsive electrostatic interactions between residues at these positions destabilize the Fos oncoprotein homodimer thereby favoring Jun/Fos heterodimerization where these repulsive forces are relieved (Schüermann et al., 1991; O'Shea et al., 1992). Accordingly, obligate heterodimeric synthetic peptides have been successfully designed by including glutamic acid at all "e" and "g" positions of one peptide and lysine residues at all "e" and "g" positions of another peptide. While homodimerization was prevented in this instance, it was promoted in similar peptides containing glutamic acid and lysine residues at positions "e" and "g", respectively, by allowing the formation of interhelical salt bridges (Graddis et al., 1993; O'Shea et al., 1992; Zhou et al., 1994).

3.3– Stages of Intermediate Filament *In vitro* Assembly

3.3.1– Formation Of A Parallel, In Register, Coiled-Coil Dimer

As mentioned previously, the central rod domain of IF proteins can be subdivided into four regions containing heptad repeats of the form described above, interspersed with short linker sequences devoid of such motifs. Several lines of evidence indicate that the first step in IF assembly involves the alignment of two compatible rod domains in parallel and in axial register to generate a coiled-coil dimer. Amino acid sequencing of purified coiled-coil segments from partially proteolyzed keratin heterodimers indicated that the two

chains must be in close axial register (Wood and Inglis, 1984; Parry et al., 1985). Furthermore, computer analysis of IF protein sequences predicts that parallel and in-register arrangement of the subunits maximizes both coiled-coil formation and the number of interhelical ion pairs between the various heptad-bearing tracks in the rod domain (reviewed in Steinert and Roop, 1988). Finally, homodimers of IF protein subunits such as vimentin, desmin and NFL, which contain a unique cysteine residue in their rod domain, can be disulfide linked *in vitro* (Quinlan et al., 1986). This was considered to be possible only if the chains are in close axial register (Steinert et al., 1985; Quinlan et al., 1986).

Interestingly, IF protein rod domains contain numerous residues capable of forming α-helix stabilizing intrahelical salt bridges, which strongly suggests that such forces may also contribute to the stability of coiled-coil dimers and higher order structures (Letai and Fuchs, 1995). Although intra- and inter-helical interactions may occur independently, many residues located at positions "e" and "g" (see Fig.2b) are capable of both types of associations, leading to the notion that a switch from intrahelical to interhelical ion pairing may assist IF protein dimerization (Letai and Fuchs, 1995), as appears to be the case for the GCN4 leucine zipper (O'Shea et al., 1991).

The composition of IF protein dimers varies according to the type of filament formed. For instance, keratins are assembled from heterodimers containing a type I and a type II subunit (Hatzfeld and Weber, 1990; Steinert, 1990; Coulombe and Fuchs, 1990), while homopolymeric IFs such as vimentin and desmin are assembled from homodimers (Quinlan et al., 1986). In the case of nIF proteins, NFL homodimers have been characterized *in vitro* (Quinlan et al., 1986) and peripherin homodimers have been observed *in vivo* (Chadan et al., 1994), while α-internexin homodimer formation can be inferred from the fact that it can form homopolymeric filaments *in vivo* (Ching and Liem, 1993) and *in vitro* (Chiu et al., 1989; Balin and Miller, 1995).

3.3.2–Tetramer Formation And The Molecular Architecture Of Intermediate Filaments

The second step in IF assembly involves the formation of a tetrameric "protofilament" unit (Ahmadi and Speakman, 1978; Geisler and Weber, 1982; Quinlan et al., 1984; Lifsic and Williams, 1984; Parry et al., 1985; Geisler et al., 1985a; Ip et al., 1985a, b), as shown by sedimentation equilibrium studies and/or SDS-PAGE analysis of chemicaly crosslinked intact or partially proteolyzed tetramers.

Electron microscopy, immunoelectron microscopy, or chemical crosslinking followed by proteolysis and sequencing of crosslinked peptides have shown an anti-parallel arrangement of IF protein dimers within protofilaments (Geisler et al., 1985a; Fraser et al., 1990; Steinert, 1991a, b; Steinert et al., 1993), with the dimers being either in close axial register (Quinlan et al., 1984; Geisler et al., 1985a; Ip et al., 1985a; Hisanaga et al., 1990b; Coulombe and Fuchs, 1990), partially staggered, (Stewart et al., 1989; Fraser et al., 1990; Geisler et al., 1992; Geisler, 1993) or in both types of association (Woods and Inglis, 1984; Steinert, 1991a, b; Steinert et al., 1993). The various modes of alignment of the ~45 nm-long, parallel, in-register, IF protein coiled-coil regions within a tetramer are depicted in Fig.3. Following the terminology of Steinert et al. (1993), Fig.3b shows the A_{12} alignment where the dimers are aligned anti-parallel and in approximate register, while Figs.3c and 3d illustrate the A_{11} and A_{22} alignments where coils 1b or 2b are respectively brought into approximate register. Finally, the A_{CN} mode of interaction shown in Fig.3e involves a 1-3 nm overlap of the C- and N-terminal ends of the rod domains. These alignments allow various surface lattice models of IF architecture to be generated (Steinert et al., 1993; Heins et al., 1993). In these models, the linker regions are predicted to create a series of gaps while the A_{CN} overlaps, and probably the tail domains of IF proteins, are predicted to create knobs at regular intervals along the filament. These regularly distributed features would have a spacing of ~21 nm (~1/2 the length of the rod, minus the A_{CN} overlap), and may already be apparent at the tetrameric protofilament or

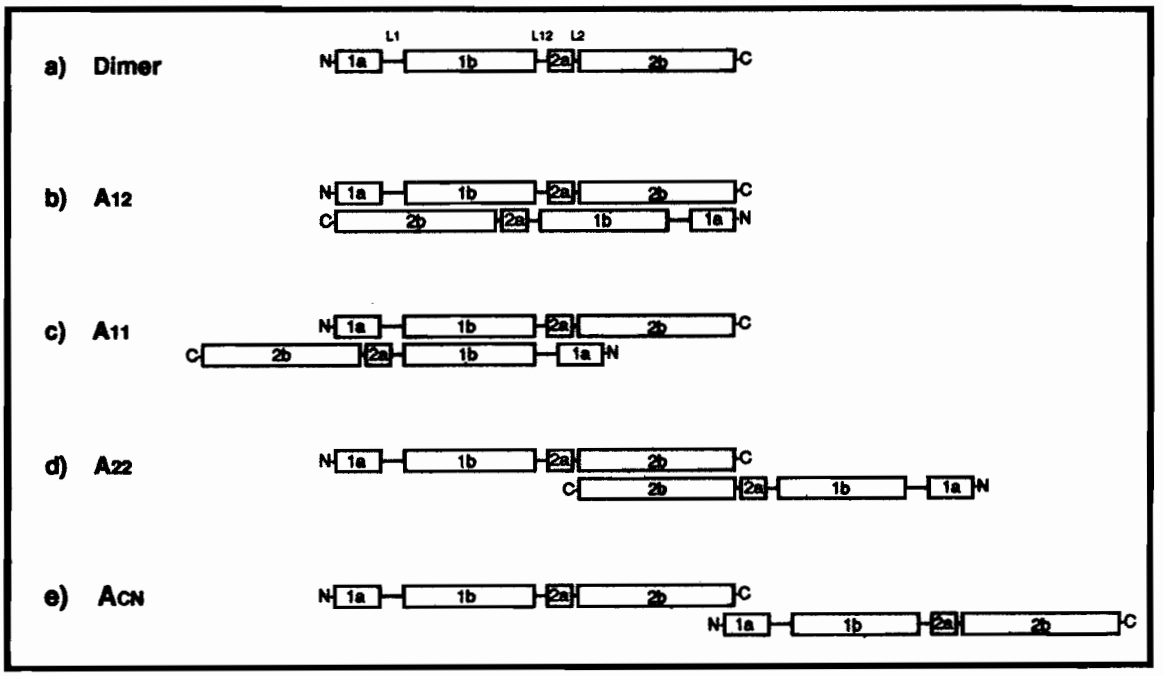

Figure 3: Diagrammatic representation of the various modes of alignment of coiled coil dimers. a) One parallel, unstaggered, coiled coil dimer. The amino- (N) and carboxy- (C) terminals have been indicated but the head and tail domains have been omitted for simplicity. The four coil regions (1a, 1b, 2a, and 2b) and the three linker regions (L1, L12 and L2) are shown. b) A_{12}, head to tail, unstaggered arrangement of two coiled coil dimers in a tetramer. c) A_{11}, head to tail arrangement with the coil 1 segments approximately in register. d) A_{22}, head to tail arrangement with the coil 2 segments approximately in register. e) A_{CN}, head to tail arrangement with a short 1-3 nm overlap. References are cited in the text.

octameric protofibril stage (Heins et al., 1993). Such features have been proposed to account for the ~21 nm beading motif (Ip et al., 1985b, Geisler et al., 1985a; Steinert et al., 1993; Heins et al., 1993) frequently observed in whole or protofilamentous IFs sprayed with glycerol and visualized by electron microscopy after rotary shadowing (Henderson et al., 1982; Milam and Erickson, 1982; Aebi et al., 1983).

Although surface lattice models can account for many features of IF structure, they require that the arrangement described be wrapped around a cylinder in order to obtain a three dimensional view of the filament (reviewed in Fraser et al., 1990). Such wrapping implies that IFs are essentially hollow, tube-like structures, which is inconsistent with the unraveling of whole filaments into subfilamentous structures frequently observed by electron microscopy (Aebi et al., 1983; Ip et al., 1985b; Hisanaga and Hirokawa, 1990b; Fraser et al., 1990; Heins et al., 1993). Furthermore, radial density estimates of keratin and type III IFs by dark field scanning transmission electron microscopy analysis attest that they contain a non-hollow core (Steven, 1990, and references therein). Scanning transmission electron microscopy measurements of average mass per length further indicate that IFs are heterogeneous in size, even within a given filament, and imply that they contain between 24 and 42 polypeptides in cross-section, the more common value being 32 (i.e., 8 protofilaments) (Steven et al., 1983; Engel et al., 1985; Heins et al., 1993). The fact that the linear densities of these polymorphic variants are separated by simple integral intervals is readily accounted for if one assumes that IFs are assembled from variable numbers of subfilaments (Steven, 1990). Taken together, these and other findings strongly indicate that IF proteins are packed into subfilamentous structures intertwined in some yet undefined way to generate a whole filament, in a manner analogous to that proposed by Fuchs and Weber (1994).

3.4– *In Vivo* Assembly Of Intermediate Filaments

A number of *in vivo* studies have been conducted where cDNAs encoding IF proteins are transfected into cells of various endogenous IF phenotype. The resulting immunofluorescence staining pattern of IF proteins is indicative of their assembly state and has revealed a number of important features pertaining to nIF assembly. For instance, the double-labeling immunofluorescent staining pattern of vimentin-containing fibroblasts transfected with NFL or NFM cDNA suggested that these subunits colocalize with the vimentin network (Chin and Liem, 1989). In the same report, immunoelectron microscopy further verified that NFM and vimentin were in fact co-assembled in the same IFs, an important observation given that co-expression of vimentin and NFs can occur during development (Bignami et al., 1982; Cochard and Paulin, 1984; Bennett and Di Lullo, 1985), or sometimes in the adult (Dräger, 1983).

More recently, transfection of the individual NF triplet proteins in a cell line devoid of endogenous IFs indicated that none of these subunits were capable of assembling by themselves, while co-transfection of NFL with either NFM or NFH resulted in a filamentous pattern, indicating that NFs are obligate heteropolymers *in vivo* (Lee et al., 1993). Ching and Liem (1993) made similar observations, and additionally pointed out that α-internexin could assemble both alone and with the individual NF subunits. Given the well-documented *in vitro* assembly capabilities of NFL (Geisler and Weber, 1981; Liem and Hutchison, 1982; Hisanaga and Hirokawa, 1990b), these findings convincingly demonstrate that additional *in vivo* elements must play an role in assembly.

3.5– Role Of The Various Domains In Neurofilament Assembly

3.5.1– Role Of The Rod Domain

The availability of cDNAs encoding NF proteins has also helped define the role of their rod, tail and head domains. Transfection of cDNAs bearing deletions extending into the highly conserved C-terminal end of the rod domain

of NFL (Gill et al., 1990), NFM (Wong and Cleveland, 1990), and NFH (Sun et al., 1993) results in the disruption of endogenous IF arrays in a "dominant" fashion, meaning that the transfected species may only represent 2-10% of the total IF content. The conserved N-terminal end of the rod domain of NFL and NFM is also essential for assembly, although its effect is considered "recessive", meaning that it is less likely to disrupt the existing network when present in low amounts (Wong and Cleveland, 1990; Lee et al., 1993).

3.5.2– Role Of The Tail Domain

The tail domain of NFM was found to be dispensable for its coassembly with vimentin or NFL (Wong and Cleveland, 1990; Ching and Liem, 1993; Lee et al, 1993; Nakagawa et al., 1995), although some bundling of the resulting filaments could be observed. On the other hand, the tail domain of NFL has been variously found to be indispensable (Gill et al., 1990) or not essential (Ching and Liem, 1993) for assembly, and can be substituted for by the NFH tail domain in transfected cells (Lee et al.,1993). Interestingly, the NFH tail domain was necessary for its assembly with NFL but not with vimentin (Sun et al., 1997).

3.5.3– Role Of The Head Domain

The head domains of NFL (Gill et al., 1990; Lee et al., 1993), NFM (Lee et al. 1993), and NFH (Sun et al., 1993) are usually found to be essential for assembly, although extensive truncations in the head domain of NFM can be accomodated in some cases (Wong and Cleveland, 1990).

3.5.4– Conclusions And Perspectives

It should be pointed out that several of the described mutants were considered assembly competent in the sense that they were readily incorporated into the existing IF network in transfected cells. However, larger accumulations of "assembly competent" truncated proteins can lead to disruption of IF arrays (Gill et al., 1990; Ching and Liem, 1993) and, in that sense, all three domains of NF proteins can be said to participate in assembly.

Moreover, the cell lines used for transfection may not faithfully duplicate the subtle necessities of NF transport and assembly in an actual neuron, such that additional functions of the head and tail domains may yet be discovered.

Though useful in defining domains that are essential for assembly, these *in vivo* studies still fail to delineate their precise function. In the case of NFL, an attempt has been made to solve this question. *In vitro* reassembly of recombinant NFL bearing various deletions indicated that the head domain promotes lateral associations of protofilaments into higher order structures while the tail domain controls the lateral assembly of protofilaments so that it stops when the filaments reaches ~10 nm in diameter (Heins et al., 1993).

4– Dynamics Of Intermediate Filaments

4.1– Intermediate Filaments Are Dynamic Structures *In Vivo*

IFs were first considered to be essentially static structures within the cell. This belief was mainly rooted in the observation that they are largely insoluble in physiological buffers containing non-ionic detergents, and that the soluble pool of their subunit constituents *in vivo* is usually rather small. As reviewed in the following section, IFs can actually be rather dynamic entities capable of various types of reorganization.

One early line of evidence pointing to the dynamic nature of IFs came from the differential rearrangement of coexisting keratin and vimentin networks in PtK2 cells (Aubin et al., 1980) and the assembly/disassembly cycles displayed by nuclear lamins during mitosis (Gerace and Blobel, 1980). Furthermore, some IF networks were shown to collapse upon microinjection of specific antibodies against vimentin (Gawlitta et al., 1981), keratin (Eckert and Daley, 1981), or the universally conserved epitope (Pruss et al, 1980) of IF proteins (Klymkowski, 1981), indicating that dynamic IF protein assembly intermediates were likely being trapped by the antibodies. Additionally, IF networks are typically reorganized during viral infection (Ball and Singer, 1981)

or heat shock (Thomas et al., 1981). The notion that IFs are dynamic structures is now well established and a number of recent studies have shed more light on the mechanisms involved.

4.1.1– Microinjection Studies

A convincing demonstration of the dynamic nature of IF networks in living cells came from microinjection experiments with soluble IF proteins tagged with biotin or fluorescent labels. An initial study showed that upon microinjection into fibroblasts, biotinylated vimentin first formed punctate aggregates, and then accumulated in the juxtanuclear region before redistributing throughout the cytoplasm, implying the presence of an IF "organizing center" (Vikstrom et al., 1989). Moreover, the endogenous filaments and the network eventually formed by the injected subunits could not be distinguished by double label immuno-fluorescence microscopy. Incidentally, "organizing centers" have also been reported in some transfection experiments (e.g., Chin and Liem, 1989; Gill et al., 1990), but are by no means universal among IFs since incorporation of new subunits can also occur without passing through such structures (reviewed in Steinert and Liem, 1990).

In a second set of experiments, microinjected rhodamine-labelled vimentin was first allowed to assemble before fluorescence recovery after photobleaching experiments were carried out. Discrete zones, bleached with a finely focused laser beam, were shown to recover their fluorescence with $t_{1/2} \approx 40$ min, presumably through exchange and incorporation of soluble fluorescent intermediates (Vikstrom et al., 1992). The lack of polarity in the recovery pattern suggested that new subunits were incorporated along the entire length of the filaments. Similar results were obtained in cultured neurons using labeled NFL (Okabe et al., 1993; Takeda et al., 1994) or NFH (Takeda et al., 1994), and immunoelectron microscopic examination of these cultures clearly demonstrated that incorporation of new subunits occurred randomly along the filaments. Intriguingly, fluorescence recovery was faster in growing axons than in quiescent ones (Okabe et al., 1993), and NFH recovered almost

twice as rapidly as NFL (Takeda et al., 1994), indicating that the incorporation process is somehow regulated. The lack of polarity in the incorporation pattern of new subunits is consistent with current models of IF architecture where antiparallel arrangement of the subunits results in an overall apolar structure (see above).

4.1.2– Phosphorylation Modulates Neuronal Intermediate Filament Assembly In Vivo

As alluded to earlier, IFs may undergo extensive reorganization during mitosis. This phenomenon was shown to be associated with IF protein phosphorylation (Evans and Fink, 1982; Ottaviano and Gerace, 1985; Dessev and Goldman, 1988; Chou et al., 1989) involving, in some cases, the cell cycle-regulated $p34^{cdc2}$ kinase (Chou et al., 1990; Peter et al., 1990; also reviewed in Skalli and Goldman, 1991, and Fuchs and Weber, 1994).

Another striking example of assembly being modulated by *in vivo* phosphorylation can be seen when cultured embryonic dorsal root ganglion (DRG) neurons are treated with the shellfish toxin, okadaic acid. This potent protein phosphatase 1 and protein phosphatase 2a inhibitor (Cohen et al., 1989; Ishihara et al., 1989) rapidly induces NF disassembly into Triton-soluble oligomers (Sacher et al., 1992, 1994; Giasson et al, 1996). The effect is reversible to a point (Sacher et al., 1992), and is associated with an increase in NFL head domain phosphorylation (Sacher et al., 1994; Giasson et al., 1996). On the other hand, inhibition of protein kinase C in neuronal cultures was shown to reverse NF aggregation, indicating that phosphorylation can impact on NF homeostasis in different ways (Carter et al., 1996).

4.1.3– Identification Of Phosphorylation Sites Pertinent To The In Vivo *Assembly Of NFL*

Sequence analysis of phosphopeptides initially identified Ser^{55} as the major protein kinase A (PKA) phosphorylation site in the head domain of NFL. Phosphorylation of Ser^{55} was thus assumed to be responsible for NF

disassembly, an attractive hypothesis consistent with its rapid turnover *in vivo* (Sihag and Nixon, 1991). This initial report overlooked an anomalously migrating peptide which contained Ser^{2}, another phosphorylation site for PKA whose *in vivo* phosphorylation state correlated more closely with NF disassembly (Giasson et al., 1996). Furthermore, wild-type and mutant NFL in which Ser^{55} was mutated to alanine or aspartate to either prevent or mimic permanent phosphorylation, respectively, were capable of assembling with the endogenous IF network upon transfection of vimentin-containing cells (Gibb et al., 1996). In IF^{-} cells, where NFL is known not to assemble on its own (Lee et al., 1993; Ching and Liem, 1993), Gibb et al. (1996) observed that the aggregation pattern produced by the Asp^{55} mutant differed from the similar appearing wild-type and Ala^{55} mutants, indicating that Ser^{55} played a subtle role in NF assembly. Taken together, these results suggest that both Ser^{2} and Ser^{55} are important for *in vivo* assembly of NFL, although their precise contribution remains to be elucidated.

4.2– *In Vitro* Dynamics Of Neurofilaments

4.2.1– Neurofilaments May Show Dynamic Behaviour In Vitro

Whether or not NFs are also dynamic entities *in vitro* is somewhat controversial. For instance, an initial report showed that a dynamic equilibrium existed between a small soluble pool of NFL and reassembled homopolymeric filaments, as monitored by resonance energy transfer of fluorescently labeled subunits (Angelides et al., 1989). In a more recent report, however, such dynamic exchange was not detected between filaments reassembled from the NF triplet proteins and a pool of soluble precursors, suggesting perhaps that *in vitro* exchange of subunits is limited to NFL homopolymeric systems (Nash and Carden, 1995).

4.2.2– Phosphorylation Modulates Neurofilament Assembly In Vitro

Much as was the case for vimentin (Evans, 1988; Geisler et al., 1989) and desmin (Geisler and Weber, 1988), *in vitro* phosphorylation by protein

4.2.2– Phosphorylation Modulates Neurofilament Assembly In Vitro

Much as was the case for vimentin (Evans, 1988; Geisler et al., 1989) and desmin (Geisler and Weber, 1988), *in vitro* phosphorylation by protein kinase C (PKC) (Hisanaga et al, 1990a; Gonda et al., 1990) and PKA (Hisanaga et al, 1990a; Nakamura et al., 1990) has been shown to render NFL homooligomers incapable of reassembly. Furthermore, *in vitro* phosphorylation of either NFL or NFM with PKA prevented their coassembly into pelletable filaments, while still allowing heterotetrameric associations to take place (Streifel et al., 1996). NFL homopolymeric filaments also disassemble upon PKA (Nakamura et al., 1990) or PKC (Gonda et al., 1990) treatment, indicating that the relevant phosphorylation sites are accessible *in situ*. Interestingly, however, a more recent report by Hisanaga et al. (1994) showed that NFs reassembled from the three subunits were also disassembled by PKA treatment, albeit at much lower levels than NFL homopolymers, whereas native NFs were not. Instead, electron microscopic examination showed that PKA-induced phosphorylation of native NFs led to thinning or severing of the filaments and quantification showed that reassembled NFs could incorporate ~three-fold more phosphate than native NFs upon PKA treatment. Hence, there appear to be some differences between the two types of filaments, perhaps due to structural changes induced by urea denaturation of the subunits used for reassembly, or to the presence of intact NF-associated proteins in the native preparations (Hisanaga et al., 1994). Nevertheless, *in vitro* phosphorylation of NFs does have some impact on their assembly, and further examination again implicated NFL head domain phosphorylation in the modulatory effect (Gonda et al., 1990; Hisanaga et al., 1994).

4.3–Axonal Transport Of Neurofilament Proteins

Given that most of the protein synthetic machinery is located in the neuronal cell body, maintenance of the axonal cytoskeleton must rely on axonal transport to provide the necessary cytoskeletal constituents. By pulse-radiolabeling neuronal cell bodies and following the fate of newly synthesized

proteins as they migrate down the axon, two broad sets of data have emerged concerning axoplasmic transport of NF proteins. In early studies carried out in large, myelinated, peripheral axons, NF proteins appeared to migrate down the axons as a coherent, non-spreading wave, with a velocity of 1.0-1.2 mm/day, referred to as the slow component a (or group V) axonal transport (Hoffman and Lasek, 1975; Black and Lasek, 1980; Hoffman et al, 1984, 1985; also Lasek et al., 1992 and references therein). The additional finding that the majority of axonal NF proteins are assembled (as opposed to soluble) (Morris and Lasek, 1982; Black et al., 1986) has led to the proposal that NF proteins are transported down the axon as assembled polymers that slide past one another (Lasek, 1986; Lasek et al., 1992).

Turning to the optic nerve and carrying out longer-term, follow-up studies on the fate of pulse-radiolabeled NF proteins, Nixon and Logvinenko (1986) reported the presence of an essentially stationary pool of labeled NF proteins in equilibrium with a mobile phase that migrated along axons at 0.5-0.7 mm/day. Furthermore, the most highly phosphorylated forms of NFH were found to be associated with the stationary pool of NF proteins, while the less phosphorylated species were more dynamic in nature (Lewis and Nixon, 1988). In addition, injection of mouse retina with both ^{32}Pi and ^{35}S-methionine labeled separate pools of NFs (Nixon et al., 1994). Interestingly, the ^{32}Pi label quickly spread over the entire nerve segment under study, became incorporated into a mostly stationary pool of NFs, and showed little anterograde translocation and very slow turnover. Meanwhile 60% of the ^{35}S label was associated with the mobile phase of newly synthesized subunits, as reported previously (Nixon and Logvinenko, 1986). Collectively, these findings firmly established that, at least in some systems, there is a stationary but dynamic pool of NF proteins in equilibrium with a more mobile phase (Nixon and Logvinenko, 1986; Nixon, 1991; Nixon et al., 1994). Such a model is consistent with the previously described fluorescence recovery after photobleaching studies of Okabe et al. (1993) and Takeda et al. (1994) involving microinjection of fluorescently-labeled NF subunits (see above). These authors failed to

detect any anterograde translocation of bleached zones within assembled filaments, and the random appearance of injected subunits along the filament length could best be accounted for by lateral incorporation of new subunits (Okabe et al., 1993; Takeda et al., 1994). Such a model is also compatible with a recent report indicating that NFM was transported along MTs in an unpolymerized form in a transgenic mouse line lacking axonal NFs (Terada et al., 1996).

It should be mentioned that multiple kinetic pools of NFs could, in principle, account for both types of data if one assumes that in some instances, the mobile pool of NF protein is very large (Nixon and Logvinenko, 1986). To that end, it is interesting to note that a small but detectable trail of NF proteins can be observed after the passage of the main radiolabeled wave in many studies of axoplasmic transport (Nixon and Logvinenko, 1986, and references therein; Hollenbeck, 1989; Nixon, 1991; also see Fig.1 in Hoffman and Lasek, 1975).

5– Transgenic Mouse Lines With Altered Neurofilament Protein Expression

5.1– Neurofilament Accumulation In Neurodegenerative Diseases

Abnormal accumulations of NFs have been observed in particular areas of neurons affected by a variety of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (reviewed in Vickers et al., 1994) as well as Creutzfeldt-Jacob disease (Liberski et al., 1995), and amyotrophic lateral sclerosis (ALS) (Carpenter, 1968; Hirano et al., 1984a, b; Schmidt et al., 1987). ALS remains a poorly understood neurodegenerative disease involving large myelinated motor neurons which progressively degenerate and die, eventually leading to paralysis and death of the patient (Banker, 1986; Chou, 1992). NFs frequently accumulate in the perikaryon and proximal axons of motor neurons in ALS patients (Carpenter, 1968; Hirano et al., 1984a, b; Schmidt et al., 1987).

As with the previously mentioned neurodegenerative diseases, it is unclear whether these NF accumulations are a cause or consequence of ALS. However, recent studies involving transgenic animals with altered NF subunit expression have shed more light on the possible involvement of NFs in the pathogenesis of ALS.

5.2–Transgenic Mouse Models Of ALS

Initial studies of transgenic mice overexpressing human (Julien et al., 1987) or murine (Monteiro et al., 1990) NFL failed to uncover any obvious neurological phenotype, although the latter showed an increase in NF density without any concomitant increase in axonal caliber. In subsequent reports, two to four-fold overexpression of human NFH (Côté et al., 1993) or murine NFL (Xu et al., 1993) resulted in perikaryal and proximal accumulation of NFs. This caused atrophy of distal axons (Côté et al., 1993; Collard et al., 1995) and increased axonal degeneration of DRGs and anterior horn motor neurons (Xu et al., 1993). In both cases, the morphological changes induced by NF overexpression resembled those seen in ALS, and were accompanied by progressive terminal paralysis of the transgenic animals (Côté et al., 1993; Xu et al., 1993). However, another hallmark of ALS, selective, extensive motor neuron death, was not observed in these animals, but did occur in transgenic mice expressing a mutant form of NFL likely to disrupt NF assembly (Lee et al., 1994). Taken together, these studies suggested that NF disorganization stemming from altered subununit stoichiometries or inappropriate assembly can contribute to the aetiology of motor neuron diseases (Côté et al., 1993; Xu et al., 1993; Lee et al., 1994; Julien, 1995).

Two mechanisms linking NF accumulations to neuronal cell death have been elucidated. In the human NFH-expressing transgenic mice, Collard et al. (1995) attributed the pathological effect of NF accumulation in the proximal axon to a reduction in axonal transport of cytoskeletal elements, mitochondria and other components necessary for axonal maintenance. An alternate pathway appeared to be at work in a transgenic line that expresses an NFH/β-

numerous cellular organelles. These latter neurons all degenerated within 18 months following birth, suggesting that entrapment of cellular organelles within perikarya can also result in neuronal cell death (Tu et al., 1997).

Mechanisms other than overexpression or mutation of NF subunits can lead to abnormal NF accumulations and concomitant ALS-like symptoms in human patients or transgenic animals. Of the 10% of ALS cases that are hereditary, one-fifth involve a dominant missense mutation in superoxide dismutase I (SOD-1) (Rosen et al., 1993) that appears to result in the gain of a toxic function (Gurney et al., 1994; reviewed in Bruijn and Cleveland, 1996). Interestingly, both SOD-1 mutation and massive accumulation of NFs in neuronal perikarya and proximal axons have been observed in at least one ALS patient (Rouleau et al., 1996). Moreover, transgenic mice expressing ALS-causing SOD-1 mutant proteins exhibit motor-neuron disease symptoms, including NF accumulation (Gurney et al., 1994, Tu et al., 1996). Taken together, these and other data suggest that the toxic effect of SOD-1 mutations may be manifested in the formation of disease-causing NF accumulations (Bruijn and Cleveland, 1996).

The precise mechanism whereby altered NF expression or SOD-1 mutation leads to NF accumulation remains speculative. In NF transgenic animals that overexpress normal NF subunits (Côté et al., 1993, Xu et al., 1993) or mutant NFL (Lee et al., 1994), altered NF subunit ratios or disruption of NF assembly somehow translates into accumulation of NFs, perhaps due to precipitation of unassembled subunits. In the case of SOD-1 mutation, the toxic function suspected to be gained may be the catalysis of tyrosine nitration (Crow et al., 1997a), of which NFL is the principal target in brain extracts (Crow et al., 1997b). This latter finding is significant, considering that nitrated NFL disrupts NF assembly *in vitro,* and may thus lead to NF accumulation (Crow et al., 1997b).

5.3– Towards A Better Understanding Of Neurofilament Function

One of the more vexing questions facing neurobiologists concerns the actual function of NFs in neurons. In this regard, recent studies confirm that NFs are a major determinant of axonal caliber as proposed previously by Hoffman et al. (1984; 1985; 1987). Indeed, a mutant quail that expresses a truncated version of NFL and fails to accumulate NFs in axons showed a markedly reduced caliber of myelinated axons (Yamasaki et al., 1991; 1992; Osamu et al., 1993). This was accompanied by a reduction in conduction velocity presumably responsible for the observed trembling phenotype (Sakaguchi et al., 1993). Moreover, expression of an NFH/β-galactosidase fusion protein (Eyer and Peterson, 1994) and targeted gene disruption of the NFL gene (Zhu et al., 1997) both resulted in a lack of axonal NFs, with a concomitant reduction in myelinated axon caliber. In addition, the latter mice unexpectedly showed delayed axonal regeneration following sciatic nerve crush, although the precise mechanism by which NFs contribute to regeneration remains speculative (Zhu et al., 1997). Although a role for NFs in maintenance of axonal caliber and possibly in axonal regeneration are the best documented putative functions so far, further research in the field may yet uncover other, still unsuspected functions.

6– Objectives And Rationale Of The Work Presented

6.1– Objectives

1) To test whether cultured DRG neurons contained α-internexin in addition to the NF proteins and peripherin, and to determine their developmental and localization patterns of expression.
2) To test whether α-internexin and peripherin were also susceptible to okadaic acid-induced disassembly and compare the time course with that observed for NF proteins.

3) To determine the composition of solubized nIF fragments resulting from okadaic acid-induced depolymerization.
4) To establish reassembly and semi-native gel electrophoresis conditions for the study of small nIF assembly intermediates.
5) To determine the composition of the smallest unit of heteromeric nIF protein assembly.
6) To determine the relative stability of the NFL/NFL, NFH/NFL and NFM/NFL dimers.
7) To assess whether the relative stability of these dimers is affected by a)post-translational modifications typically found in spinal cord NFL or b) a mutation suspected of affecting NF assembly.

6.2– Rationale Behind The Work Presented

Two new members of the nIF protein family had recently been described. α-Internexin was found throughout the nervous system, and was especially prominent early in development (Kaplan et al., 1990; Fliegner et al., 1990) while peripherin was found in neurons that project to the periphery (Portier et al., 1984, Escurat et al., 1990; Goldstein et al., 1991). Interestingly, the expression of α-internexin and peripherin overlapped with that of NF proteins during development and in a subset of adult neurons, including some DRG neurons (reviewed by Nixon and Shea, 1992). Hence, mammalian nIFs could be composed of as many as five different proteins. This raised questions concerning the level at which coassembly was taking place. Previous work in our laboratory had demonstrated that the shellfish toxin okadaic acid triggers NF disassembly in cultured DRGs (Sacher et al., 1992). Furthermore, pilot studies showed that mAbs specific for the individual NF subunits could co-immunoprecipitate other NF subunits in extracts from okadaic acid-treated neurons. Since mAbs specific for α-internexin and peripherin had recently become commercially available, this suggested a way to probe the nature of

disassembly intermediates as a means to further understand heteropolymeric assembly of the five nIF subunits.

Having observed extensive co-immunoprecipitation of nIF proteins in this system, the nature and composition of the smallest nIF protein heterooligomers was further investigated. To this end, the recently described high resolution, non-denaturing gel system of Schägger et al. (1994) was adapted to the study of heteromers partially assembled from purified nIF subunits.

Having determined that most nIF subunit combinations led to heterodimer formation, the relative stability of some of these dimers was investigated as a means to better understand assembly-related problems. Specifically, some posttranslational modifications such as phosphorylation in the head domain of NFL had been shown to promote its disassembly *in vivo* (Sacher et al., 1994; Giasson et al., 1996) and *in vitro* (Hisanaga et al., 1990a; Gonda et al., 1990). Moreover, a mutation in NFL suspected to affect NF assembly was shown to cause NF accumulation in a transgenic mouse model whose symptoms were reminiscent of motor neuron disease (Lee et al., 1994). In both instances, there is a general lack of understanding regarding the mechanism by which posttranslational modification or mutation influence assembly. The techniques developed during the course of this work were thus used to assess their impact on NF dimer stability as a means to further our understanding of NF assembly.

REFERENCES

Adams, R.J. (1982) Organelle movement in axons depends on ATP. *Nature* **297**, 327-329.

Aebi, U., Fowler, W.E., Rew, P. and Sun, T.-T. (1983) The fibrillar structure of keratin filaments unravelled. *J. Cell Biol.* **97**, 1131-1143.

Ahmadi, B. and Speakman, P.T. (1978) Suberimidate cross-linking shows that a rod-shaped, low-cystine, high-helix protein prepared by limited proteolysis of reduced wool has four protein chains. *FEBS Lett.* **94**, 365-367.

Allan, V.J., Vale, R.D. and Navone, F. (1991) Microtubule-based organelle transport in neurons. *The Neuronal Cytoskeleton* (Burgoyne, R.D., editor), pp 257-282, Wiley-Liss, New-York.

Angelides, K.J., Smith, K.E. and Takeda, M. (1989) Assembly and exchange of intermediate filament proteins of neurons: neurofilaments are dynamic structures. *J. Cell Biol.* **108**, 1495-1506.

Aubin, J. E., Osborn, M., Franke, W. W. and Weber, K. (1980) Intermediate filaments of the vimentin-type and the cytokeratin-type are distributed differently during mitosis. *Exp. Cell Res.* **129**, 145-165.

Baas, P.W. (1997) Microtubules and axonal growth. *Curr. Opin. Cell Biol.* **9**, 29-36.

Baas, P.W., Pienkowski, T.P. and Kosik, K.S. (1991) Processes induced by tau expression in Sf9 cells have an axon-like microtubule organization. *J. Cell Biol.* **115**, 1333-1344.

Baines, A.J. (1991) Neuronal plasma membrane associated cytoskeleton. *The Neuronal Cytoskeleton* (Burgoyne, R.D., editor), pp 161-183. Wiley-Liss, New-York.

Balin, B.J. and Lee, V.M.-Y. (1991) Individual neurofilament subunits reassembled *in vitro* exhibit unique biochemical, morphological and immunological properties. *Brain Res.* **556**, 196-208.

Balin, B.J. and Miller, M.E. (1995) Reassembly of the 66 KDa neurofilament protein *in vitro* following isolation and purification from bovine spinal cord. *J.Neurosci.Res.* **40**, 79-88

Balin, B.J., Clark, E.A., Trojanowski, J.Q. and Lee, V.M.-Y. (1991) Neurofilament reassembly *in vitro*: biochemical, morphological and immuno-electron microscopic studies employing monoclonal antibodies to defined epitopes. *Brain Res.* **556**, 181-195.

Ball, E.H. and Singer, S.J. (1981) Association of microtubules and intermediate filaments in normal fibroblasts and its disruption upon transformation by a temperature-sensitive mutant of Rous sarcoma virus. *Proc. Nat. Acad. Sci. USA* **78**, 6986-6990.

Bamburg, J.R. and Bernstein, B.W. (1991) *The Neuronal Cytoskeleton* (Burgoyne, R.D., editor), pp 121-160. Wiley-Liss, New-York.

Banker, B.Q. (1986) *Myology: the pathology of the motor neuron diseases* (Engel, A.G and Banker, B.Q., editors), pp 2031-2066. McGraw-Hill, New-york.

Barra, H.S., Arce, C.A., Rodriguez, J.A. and Caputto, R. (1974) Some common properties of the protein that incorporates tyrosine as a single unit and the microtubule proteins. *Biochem. Biophys. Res. Commun.* **60**, 1384-1390.

Barra, H.S., Rodriguez, J.A., Arce, C.A. and Caputto, R. (1973) A soluble preparation from rat brain that incorporates into its own proteins [^{14}C]-arginine by a ribonuclease-sensitive system and [^{14}C]-tyrosine by a ribonuclease-insensitive system. *J. Neurochem.* **20**, 97-108.

Bennet, G. S. and Di Lullo, C. (1985) Transient expression of a neurofilament protein by replicating neuroepithelial cells of the embryonic chick brain. *Dev. Biol.* **107**, 107-127.

Berthold, C.H. (1978) Morphology of normal peripheral axons. *Physiology and pathobiology of axons* (S.G. Waxman, editor), pp 3-63, Raven Press, New-York.

Bignami, A., Rajn, T. and Dahl, D. (1982) Localization of vimentin, the nonspecific intermediate filament protein, in embryonal glia and in early differentiating neurons. *Dev. Biol.* **91**, 281-295.

Binder, L.I., Frankfurter, A. and Rebhun, L.I. (1985) The distribution of Tau in the mammalian central nervous system. *J. Cell Biol.* **101**, 1371-1378.

Black, M.M. and Lasek, R.J. (1980) Slow components of axonal transport: two cytoskeletal networks. *J. Cell Biol.* **86**, 616-623.

Black, M.M., Keyser, P. and Sobel. E. (1986) Interval between the synthesis and assembly of cytoskeletal proteins in cultured neurons. *J. Neurosci.* **6**, 1004-1012.

Blikstad, I, Sundkvist, I. and Eriksson, S. (1980): Isolation and characterization of profilactin and profilin from calf thymus and brain. *Eur. J. Biochem.* **105**, 424-434.

Bloom, G.S., Luca, F.C. and Vallee, R.B. (1984a) Widespread cellular distubution of MAP 1A (microtubule-associated protein 1A) in the mitotic spindle and on interphase microtubules. *J. Cell Biol.* **98**, 331-340.

Bloom, G.S., Schoenfeld, T.A. and Vallee, R.B. (1984b) Widespread cellular distubution of MAP 1 (microtubule-associated protein 1) in the nervous system. *J. Cell Biol.* **98**, 320-330.

Bloom, G.S., Wagner, M.C., Pfister, K.K., Leopold, P.I. and Brady, S.T. (1989) Involvement of microtubules and kinesin in the fast axonal transport of membrane-bounded organelles. *Cell Movement, Vol.2: Kinesin, Dynein, And Microtubule Dynamics* (Warner, F.D. and McIntosh, J.R., editors) pp 321-333. Alan R. Liss Inc., New-York.

Borisy, G.G., Marcum, J.M, Olmsted, J.B., Murphy, D.B. and Johnson, K.A. (1975) Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly *in vitro. Ann. N.Y. Acad. Sci.* **253**, 107-132.

Bornslaeger, E.A., Corcoran C.M., Stappenbeck, T.S. and Green, K.J. (1996) Breaking the connection: displacement of the desmosomal plaque protein desmoplakin from cell-cell anchorage interfaces disrupts anchorage of

intermediate filament bundles and alters intercellular junction assembly. *J. Cell Biol.* **134**, 985-1001.

Brady, , S.T., Lasek, R.J. and Allen, R.D. (1982) Fast axonal transport in extruded axoplasm from squid giant axon. *Science* **218**, 1129-1131.

Brown, A. and Lasek, R.J. (1988) Neurofilaments are inherently non-interactive and will disperse radially in the absence of an axonal plasma membrane. *J. Cell Biol.* **107**, 464a.

Brown, A., Dalpe, G., Mathieu, M. and Kothary, R. (1995) The mouse dystonia musculorum gene is a neural isoform of bullous pemphigoid antigen 1. *Nature Genet.* **10**, 301-306.

Bruijn, L.I. and Cleveland, D.W. (1996) Mechanism of selective motor neuron death in ALS: insights from transgenic mouse models of motor neuron disease. *Neuropathol. Appl. Neurobiol.* **22**, 373-387.

Burton, P.R. and Paige, J.L. (1981) Polarity of axoplasmic microtubules in the olfactory nerve of the frog. *Proc. Natl. Acad. Sci. USA* **78**, 3269-3273.

Caceres, A. and Kosik, K.S. (1990) Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. *Nature* **343**, 461-463.

Caceres, A., Mautino, J. and Kosik, K.S. (1992) Suppression of MAP2 in cultured cerebellar macroneurons inhibits minor neurite formation. *Neuron* **9**, 607-618.

Carden, M.J. and Eagles, P.A.M. (1983) Neurofilaments from ox spinal nerves. *Biochem J.* **215**, 227-237.

Carden, M.J., Trojanowski, J.Q., Schlaepfer, W.W. and Lee, V.M.-Y. (1987) Two stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J. Neurosci.* **7**, 3489-3504.

Carlier, M.F. and Pantaloni, D. (1997) Control of actin dynamics in cell motility. *J. Mol. Biol.* **269**, 459-467.

Carpenter, D.A. and Ip, W. (1996) Neurofilament triplet protein interactions: evidence for the preferred formation of NFL containing dimers and a putative function for the end domains. *J. Cell Science* **109**, 2493-2498.

Carpenter, S. (1968) Proximal axonal enlargement in motor neuron disease. *Neurology (Minneap.)* **18**, 841-851.

Carter, J.E., Gallo, J.M., Anderson, V.E.R., Anderton, B.H. and Robertson, J. (1996) Aggregation of neurofilaments in NFL transfected neuronal cells: regeneration of the filamentous network by proteine kinase C inhibitors *J. Neurochem.* **67**, 1997

Chadan, S., Moya, K.L., Portier, M.-M. and Filliatreau, G. (1994) Identification of a peripherin dimer: changes during axonal development and regeneration of the rat sciatic nerve. *J. Neurochem.* **62**, 1894-1905.

Cheek, T.R. and Burgoyne, R.D. (1991) Cytoskeleton in secretion and neurotransmitter release. *The Neuronal Cytoskeleton* (Burgoyne, R.D., editor), pp 309-325, Wiley-Liss, New-York

Chen, J., Kanai, Y., Cowan, N.J. and Hirokawa, N. (1992) Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons. *Nature* **360**, 674-677.

Chin, S.S.M. and Liem, R.K.H (1989) Expression of rat neurofilament proteins NFL and NFM in transfected non-neuronal cells. *Eur. J. Cell Biol.* **50**, 475-490.

Chin, S.S.M. and Liem, R.K.H. (1990) Transfected rat high-molecular-weight neurofilament (NF-H) coassembles with vimentin in a predominantly nonphosphorylated form. *J. Neurosci.* **10**, 3714-3726.

Chin, T.K., Harding, S.E. and Eagles, P.A.M. (1989) Characterization of two proteolitically derived soluble polypeptides from the neurofilament triplet components NFM and NFH. *Biochem. J.* **264**, 53-60.

Ching, G.Y. and Liem, R.K.H. (1993) Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. *J. Cell Biol.* **122**, 1323-1335.

Chiu, F.C., Barnes, E.A., Das, K., Haley, J., Socolow, P., Macaluso, F.P. and Fant, J. (1989) Characterization of a novel 66 Kd subunit of mammalian neurofilaments. *Neuron* **2**, 1435-1445.

Chou, C.F., Smith, A.J. and Omary, M.B. (1992) Characterization and dynamics of O-linked glycosylation of human cytokeratins 8 and 18.

Chou, S.M. (1992) Pathology: light microscopy of amyotrophic lateral sclerosis. *Hand book of amyotrophic lateral sclerosis* (Smith, R.A., editor) pp 133-181. Marcel Dekker, New-York.

Chou, Y.-H., Bishoff, J.R., Beach, D. and Goldman, R.D. (1990) Intermediate filament reorganization during mitosis is mediated by $p34^{cdc2}$ phosphorylation of vimentin. *Cell* **62**, 1063-1071.

Chou, Y.-H., Rosevear, E. and Goldman, R.D. (1989) Phoshorylation and disassembly of intermediate filaments in mitotic cells. *Proc. Natl. Acad. Sci. USA* **86**, 1885-1889.

Chrétien, D., Fuller, S.D., Karsenti, E. (1995) Structure of growing microtubule ends: two-dimensional sheets close into tubes at variable rates. *J. Cell Biol.* **129**, 1311-1328.

Cleveland, D.W., Hwo, S. and Kirschner, M.W. (1977a) Purification of tau, a microtubule-associated protein that induces assembly of microtubules, from purified tubulin. *J. Mol. Biol.* **116**, 207-225.

Cleveland, D.W., Hwo, S. and Kirschner, M.W. (1977b) Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *J. Mol. Biol.* **116**, 227-247.

Cochard, P. and Paulin, D. (1984) Initial expression of neurofilaments and vimentin in the central and peripheral nervous systems of the mouse embryo *in vivo*. *J. Neurosci.* **4**, 2080-2094.

Cohen, P., Klempp, S. and Schelling D.L. (1989) An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. *FEBS Lett.* **250**, 596-600.

Cohlberg, J.A., Hajarian, H. and Sainte-Marie, S. (1987) Discrete soluble forms of middle and high molecular weight neurofilament proteins in dilute aqueous buffers. *J. Biol. Chem.* **262**, 17009-17015.

Collard, J.-F., Côté, F. and Julien, J.-P. (1995) Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature*, **375**, 61-64.

Cole, J.S, Messing, A., Trojanowski, J.Q., and Lee, V.M.-Y. (1994) Modulation of axon diameter and neurofilaments by hypomyelinating Schwann cells in transgenic mice. *J. Neurosci.* **14**, 6956-6966.

Condeelis, J. (1993) Life at the leading edge: the formation of cell protrusions. *Annu. Rev. Cell Biol.* **9**, 411-444.

Cope, M.J., Whisstock, J., Rayment, I. and Kendrick-Jones, J. (1996) Conservation within myosin motor domain: implications for structure and function. *Structure* **4**, 969-987.

Côté, F., Collard, J.-F. and Julien, J.-P. (1993) Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. *Cell* **73**, 35-46.

Coulombe, P.A. and Fuchs, E. (1990) Elucidating the early stages of keratin filament assembly. *J. Cell Biol.* **111**, 153-169.

Crow, J.P., Ye, Y.Z., Strong, M., Kirk, M., Barnes, S. and Beckman, J.S. (1997b) Superoxide dismutase catalyzes nitration of tyrosines by peroxynitrite in the rod and head domains of neurofilament-L. *J. Neurochem.* **69**, 1945-1953.

Crow, J.P., Sampson, J.B., Zhuang, Y., Thompson, J.A. and Beckman, J.S. (1997a) Decreased Zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite. *J. Neurochem.* **69**, 1936-1944.

Crick, F.H.C. (1953) The packing of α-helices: simple coiled-coils. *Acta Crystallogr.* **6**, 689-697.

Cunningham, C.C., Leclerc, N., Flanagan, L.A., Lu, M., Janmey, P.A. and Kosik, K.S. (1997) Microtubule-associated protein 2c reorganizes both microtubules and microfilaments into distinct cytological structures in an actin-binding protein-280-deficient melanoma cell line. *J. Cell Biol.* **136**, 845-857.

Dentler, W.L, Granett, S. and Rosenbaum, J.L. (1975) Ultrastructural localization of the high molecular weight proteins associated with *in vitro* assembled brain microtubules. *J. Cell Biol.* **65**, 237-241.

Dessev, G. and Goldman, R. (1988) Meiotic breakdown of nuclear envelope in oocytes of *Spisula solidissima* involves phosphorylation and release of nuclear lamin. *Dev. Biol.* **130**, 543-550.

De Camilli P. and Greengard P. (1986) Synapsin I: a synaptic vesicle-associated neuronal phosphoprotein. *Biochem. Pharmacol.* **35**, 4349-4357.

De Waegh, S.M., Brady, S.T. and Lee, V.M.-Y. (1992) Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating schwann cells. *Cell* **68**, 451-463.

Dong, D.L.-Y., Xu, Z.-S, Chevrier, M.R., Cotter, R.J., Cleveland, D.W. and Hart, G.W. (1993) Glycosylation of mammalian neurofilaments: localization of multiple *O*-linked *N*-acetylglucosamine moieties on neurofilament polypeptides L and M. *J. Biol. Chem.* **268**, 16679-16687.

Dong, D.L.-Y., Xu, Z.-S, Hart, G.W. and Cleveland, D.W. (1996) Cytoplasmic *O*-GlcNAc modification of the head domain and the KSP repeat motif of the neurofilament protein neurofilament-H. *J. Biol. Chem.* **271**, 20845-20852.

Drager, U.C. (1983) Coexistence of neurofilaments and vimentin in a neuron of adult mouse retina. *Nature* **303**, 169-172.

Drubin, D.G. and Kirschner, M.W. (1986) Tau protein function in living cells. *J. Cell. Biol.* **103**, 2739-2746.

Eagles, P.A.M., Pant, H.C. and Gainer, H. (1990), *Cellular and molecular biology of intermediate filaments* (Goldman, R.D. and Steinert, P.M., editors) pp 37-94, Plenum Press, New York.

Edson, K., Weisshaar, B., Matus, A. (1993) Actin depolymerization induces process formation on MAP2-transfected non-neuronal cells. *Development*, **117**, 689-700.

Eipper, B.A. (1974) Properties of rat brain tubulin. *J. Biol. Chem.* **249**, 1407-1416.

Elhanany, E., Jaffe, H., Link, W.T., Sheeley, D.M., Gainer, H. and Pant, H.C. (1994) Identification of endogenously phosphorylated KSP sites in the high molecular weight rat neurofilament protein. *J. Neurochem.* **63**, 2324-2335.

Engel, A., Eichner, T. and Aebi, U. (1985) Polymorphism of reconstituted human epidermal Keratin filaments: determination of their mass per length and width by scanning transmission electron microscopy (STEM). *J. Ultrastruct. Res.* **90**, 323-335.

Ersfeld, K., Wehland, J., Plessmann, U., Dodemont, H., Gerke, V. and Weber, K. (1993) Characterization of the tubulin tyrosine ligase. *J. Cell Biol.* **120**, 725-732.

Escurat, M., Djabali, K., Gumpel, M., Gros, F. and Portier, M.-M. (1990) Differential expression of two neuronal intermediate filament proteins, peripherin and the low molecular mass neurofilament protein (NF-L), during the development of the rat. *J.Neurosci.* **10**, 764-784.

Evans, R.M. (1988) cAMP dependent protein kinase-induced vimentin disassembly involves modification of the N-terminal domain of intermediate filament subunits. *FEBS Lett.* **234**, 73-78.

Evans, R.M. and Fink, L.M. (1982) An alteration in the phosphorylation of vimentin-type intermediate filaments is associated with mitosis in cultured mammalian cells. *Cell* **29**, 43-52.

Eyer, J. and Peterson, A. (1994) Neurofilament-deficient axons and perikaryal aggregates in viable transgenic mice expressing a neurofilament–β-galactosidase fusion protein. *Neuron* **12**, 389-405.

Fath, K.R. and Lasek, R.J. (1988) Two classes of actin microfilaments are associated with the inner cytoskeleton of axons. *J. Cell Biol.* **107**, 613-621.

Fine, R.E. and Bray, D. (1971) Actin in growing nerve cells. *Nature New Biology* **234**, 115-118.

Fliegner, K.H., Ching, G.Y. and Liem, R.K.H (1990) The predicted amino acid sequence of α-internexin is that of a novel neuronal intermediate filament protein. *EMBO J.* **9**, 749-755.

Fliegner, K.H., Kaplan, M.P., Wood, T.L., Pintar, J.E. and Liem, R.K.H. (1994) Expression of the gene for the intermediate filament protein α-internexin coincides with the onset of neuronal differentiation in the developing rat nervous system. *J. Comp. Neurol.* **342**, 161-173.

Frankel, F.R. (1976) Organization and energy-dependant growth of microtubules in cells. *Proc. Natl. Acad. Sci. USA* **73**, 2798-2802.

Fraser, R.D.B., MacRae, T.P. and Parry, D.A.D. (1990) The three dimensional structure of IF. *Cellular and molecular biology of intermediate filaments* (Goldman, R.D. and Steinert, P.M., editors) pp. 205-231, Plenum Press, New York.

Frieden, C. (1985) Actin and tubulin polymerization: the use of kinetic methods to determine mechanism. *Ann. Rev. Biophys. Biophys. Chem.* **14**, 189-210.

Fuchs, E and Weber, K. (1994) Intermediate filaments: structure, dynamics, function, and disease. *Annu. Rev. Biochem.* **63**, 345-382.

Garner, C.C. and Matus, A. (1988) Different forms of microtubule-associated protein 2 are encoded by separate mRNA transcripts. *J. Cell Biol.* **106**, 779-783.

Gawlitta, W., Osborn, M. and Weber, K. (1981) Coiling of intermediate filaments induced by microinjection of a vimentin-specific antibody does not interfere with locomotion or mitosis. *Eur. J. Cell Biol.* **26**, 83-90.

Geahlen, R.L. and Haley, B.E. (1977) Interactions of a photoaffinity analog of GTP with the proteins of microtubules. *Proc. Natl. Acad. Sci. USA* **74**, 4375-4377.

Geisler, N. (1993) Chemical cross-linking with disuccinimidyl tartrate defines the relative positions of the two antiparallel coiled coils of the desmin protofilament unit. *FEBS Lett.* **323**, 63-67.

Geisler, N. and Weber, K. (1981) Self-assembly *in vitro* of the 68,000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate sized filaments. *J. Mol. Biol.* **151**, 565-571.

Geisler, N. and Weber, K. (1982) The amino acid sequence of chicken muscle desmin provides a common structural model for intermediate filament proteins. *EMBO J.* **1**, 1649-1656.

Geisler, N. and Weber, K. (1988) Phosphorylation of desmin *in vitro* inhibits formation of intermediate filaments: identification fo three kinase A sites in the aminoterminal head domain. *EMBO J.* **7**, 15-20.

Geisler, N., Fisher, S., Vandekerckhove, J., Plessmann, U. and Weber, K. (1984) Hybrid character of a large neurofilament protein (NFM): intermediate filament type sequences followed by a long acidic carboxy-terminal extension. *EMBO J.* **3**, 2701-2706.

Geisler, N., Hatzfeld, N. and Weber, K. (1989) Phosphorylation *in vitro* of vimentin by protein kinase A and C is restricted to the head domain: identification of the phosphoserine sites and their influence on filament formation. *Eur. J. Biochem.* **183**, 441-447.

Geisler, N., Kaufman, E. and Weber, K. (1985a) Antiparallel orientation of two double stranded coiled coils in the tetrameic protofilament unit in intermediate filaments. *J. Mol. Biol.* **182**, 173-177.

Geisler, N., Kaufmann, E., Fischer, S., Plessmann, U. and Weber, K. (1983) Neurofilament architecture combines structural principles of intermediate filaments with carboxy-terminal tail extensions of increasing size between triplet proteins. *EMBO J.* **2**, 1295-1302.

Geisler, N., Shünemann, J. and Weber, K. (1992) Chemical cross-linking indicates a staggered and antiparallel protofilament of desmin intermediate filaments and characterizes one higher-level complex between protofilaments. *Biochem.* **206**, 841-852.

Geisler, N., Vandekerckhove, J. and Weber, K. (1987) Location and sequence characterization of the major phosphorylation sites of the high molecular mass neurofilament proteins M and H. *FEBS Lett.* **221**, 403-407.

Geisler, N., Vandekerckhove, J., Van Damme, J., Plessmann, U. and Weber, K. (1985b) Protein-chemical characterization of NFH, the largest mammalian

neurofilament component; intermediate filament-type sequences followed by a unique carboxy-terminal extension. *EMBO J.* **4**, 57-63.

Georges, E. and Mushynski, W.E. (1987) Chemical modification of charged amino acid moieties alters the electrophoretic mobilities of neurofilament subunits on SDS-polyacrylamide gels. *Eur. J. Biochem.* **165**, 281-287.

Gerace, L. and Blobel G. (1980) The nuclear lamina is reversibly depolymerized during mitosis. *Cell* **19**, 277-287.

Giasson, B.I., Cromlish, J.A., Athlan, E.S., and Mushynski, W.E. (1996) Activation of cyclic AMP-dependent protein kinase in okadaic acid-treated neurons potentiates neurofilament fragmentation and stimulates phosphorylation of Ser-2 in the low-molecular-mass neurofilament subunit. *J. Neurochem.* **66**, 1207-1213.

Gibbs, B.J.M., Robertson, J. and Miller, C.C.J. (1996) Assembly properties of neurofilament light chain ser^{55} mutants in transfected mammalian cells. *J. Neurochem.* **66**, 1306-1311.

Gill, S.R., Wong, P.C., Monteiro, M.J. and Cleveland, D.W. (1990) Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NFL) subunit. *J. Cell Biol.* **111**, 2004-2019.

Goldberg, D.J. (1982) Microinjection into an identified axon to study the mechanism of fast axonal transport. *Proc. Natl. Acad. Sci. USA* **79**, 4818-4822.

Goldman, R.D., Khuon, S., Chou, Y.H, Opal, P. and Steinert, P.M. (1996) The function of intermediate filaments in cell shape and cytoskeletal integrity. *J. Cell Biol.* **134**, 971-983.

Goldstein, M.E., House, S.D. and Gainer, H. (1991) NF-L and peripherin immunoreactivities define distinct classes of rat sensory ganglion cells. *J.Neurosci.Res.* **30**, 92-104.

Goldstein, M.E., Sternberger, N.H. and Sternberger, L.A. (1987) Phosphorylation protects neurofilaments against proteolysis. *J. Neuroimmunol.* **14**, 149-160.

Gonda, Y., Nishizawa, K., Ando, S., Kitamura, S., Minoura, Y., Nishi. Y. and Inagaki, M. (1990) Involvement of protein kinase C in the regulation of

assembly-diassembly of neurofilaments *in vitro*. *Biochem. Biophys. Res. Commun.* **167**, 1316-1325.

Goodson, H.V., Valetti, C, and Kreis, T.E. (1997) Motors and membrane traffic. *Curr. Opin. Cell Biol.* **9**, 18-28.

Gorham, J.D., Baker, H., Kegler, D. and Ziff, E.B. (1990) The expression of the neuronal intermediate filament protein peripherin in the rat embryo. *Dev. Brain Res.* **57**, 235-248.

Gotow, T., Masatoshi, T, Tanaka, T and Hashimoto, P. H. (1992) Macromolecular structure of reassembled neurofilaments as revealed by the quick-freeze deep-etch mica method: difference between NFM and NFH subunits in their ability to form cross-bridges. *Eur. J. Cell Biol.* **58**, 331-345.

Graddis, T.J., Myszka, D.G. and Chaiken, I.M. (1993) Controlled formation of model homo- and heterodimer coiled coil polypeptides. *Biochemistry*, **32**, 12664-12671.

Green, K.J. and Jones, J.C.R. (1990) Interactions of intermediate filaments with the cell surface. *Cellular and molecular biology of intermediate filaments* (Goldman, R.D. and Steinert, P.M., editors), pp 147-174, Plenum Press, New York.

Green, K.J. and Jones, J.C.R. (1996) Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB J.* **10**, 871-881.

Greer, K. and Rosenbaum, J.L. (1989) Post-transational modifications of tubulin. *Cell Movement, Vol.2: Kinesin, Dynein, And Microtubule Dynamics* (Warner, F.D. and McIntosh, J.R., editors) pp 47-66. Alan R. Liss Inc., New-York.

Gurney, M.E., Pu, H., Chiu, A.Y. et al. (1994) Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science* **264**, 1772-1775.

Hallak, M.E., Rodriguez, J.A., Barra, H.S. and Caputto, R. (1977) Release of tyrosine from tyrosinated tubulin. Some common factors that affect this process and the assembly of tubulin. *FEBS Lett.* **73**, 147-150.

Haltiwanger, R.S., Kelly, W.G., Roquemore, E.P., Blomberg, M.A., Dong, D.L.-Y., Kreppel, L., Chou, T.-Y. and Hart, G.W. (1992) Glycosylation of nuclear and cytoplasmic proteins is ubiquitous and dynamic. *Biochem. Soc. Trans.* **20**, 264-269.

Hatzfeld, M. and Weber, K. (1990) The coiled-coil of *in vitro* assembled keratin filaments is a heterodimer of type I and II keratins: use of site specific mutagenesis and recombinant protein expression. *J. Cell Biol.* **110**, 1199-1210.

Heidemann, S.R., Landers, J.M. and Hamborg, M.A. (1981) Polarity orientation of axonal microtubules. *J. Cell Biol.* **91**, 661-665.

Heimann, R., Shelanski, M.L. and Liem, R.K.H. (1985) Microtubule-associated proteins bind specifically to the 70 KDa neurofilament protein. *J. Biol Chem.* **260**, 12160-12166.

Heins, S., Wong, P.C., Müller, S., Goldie, K. and Cleveland, D.W. (1993) The rod domain of NFL determines neurofilament architecture, whereas the end domains specify filament assembly and network formation. *J. Cell. Biol.* **123**, 1517-1533.

Henderson, D., Geisler, N. and Weber, K (1982) A periodic ultrastructure in intermediate filaments. *J. Mol. Biol.* **155**, 173-176.

Hicks, A., Davis, S., Rodger, J., Helme-Guizon, A., Laroche, S. and Mallet, J. (1997) Synapsin I and syntaxin 1B: key elements in the control of neurotransmitter release are regulated by neuronal activation and long-term potentiation *in vivo. Neuroscience* **79**, 329-340.

Hirano, A., Donnenfeld, H., Sasaki, S. and Nakano, I. (1984a) Fine structural observations of neurofilamentous changes in amyotrophic lateral sclerosis. *J. Neuropathol. Exp. Neurol.* **43**, 461-470.

Hirano, A., Nakano, I., Kurland, L.T., Mulder, D.W., Hollen, P.W. and Sacommanno, G. (1984b) Fine structural study of neurofibrillary changes in a family with amyotrophic lateral sclerosis. *J. Neuropathol. Exp. Neurol.* **43**, 471-480.

Hirokawa, N. (1982) Cross-linker system between neurofilaments, microtubules, and membranous organelles in frog axons revealed by the quick-freeze, deep-etch method. *J. Cell Biol.* **94**, 129-142.

Hirokawa, N. (1991) Molecular architecture and dynamics of the neuronal cytoskeleton. *The Neuronal Cytoskeleton* (Burgoyne, R.D., editor), pp 5-74, Wiley-Liss, New-York.

Hirokawa, N. (1994) Microtubule organization and dynamics dependent on microtubule-associated proteins. *Curr. Opin. Cell Biol.* **6**, 74-81.

Hirokawa, N., Sobue, K., Kanda, K., Harada, A. and Yorifuju, H. (1989) The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin 1. *J. Cell Biol.* **108**, 111-126.

Hirokawa, N., Bloom, G.S. and Vallee, R.B. (1985) Cytoskeletal architecture and immunocytochemical localization of microtubule-associated proteins in regions of axons associated with rapid axonal: the IDPN-intoxicated axon as a model system. *J. Cell Biol.* **101**, 227-239.

Hirokawa, N., Glicksman, M.A. and Willard, M.B. (1984) Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. *J. Cell Biol.* **98**, 1523-1536.

Hirokawa, N., Hisanaga, S.I. and Shiomura, Y. (1988a) MAP2 is a component of crossbridges between microtubules and neurofilaments in the neuronal cytoskeleton: quick-freeze, deep-etch immunoelectron microscopy and reconstitution studies. *J. Neurosci.* **8**, 2769-2779.

Hirokawa, N., Shiomura, Y. and Okabe, S. (1988b) Tau proteins: the molecular structure and mode of binding on microtubules. *J. Cell Biol.* **107**, 1449-1461.

Hisanaga, S. and Hirokawa, N. (1988) The structure of peripheral domains of the neurofilament revealed by low angle rotary shadowing. *J. Mol. Biol.* **202**, 297-306.

Hisanaga, S. and Hirokawa, N. (1989) The effect of dephosphorylation on the structure of the projections of neurofilaments. *J. Neurosci.* **9**, 959-966.

Hisanaga, S. and Hirokawa, N. (1990a) Dephosphorylation induced interactions of neurofilaments with microtubules. *J. Biol. Chem.* **265**, 21852-21858.

Hisanaga, S. and Hirokawa, N. (1990b) Molecular architecture of the neurofilament. *J. Mol. Biol.* **211**, 871-882).

Hisanaga, S., Gonda, Y., Inagaki, M., Ikai, A. and Hirokawa N. (1990) Effects of phosphorylation of the neurofilament NFL protein on filamentous structures. *Cell Regulation* **1**, 237-248.

Hisanaga, S., Ikai, A. and Hirokawa, N., J. (1990) Molecular architecture of the neurofilament. *J. Mol. Biol.* **211**, 857-869.

Hisanaga, S., Matsuoka, Y., Nishizawa, K., Saito, T., Inagaki, M. and Hirokawa, N. (1994) Phosphorylation of native and reassembled neurofilaments composed of NFL, NFM, and NFH by the catalytic subunit of cAMP-dependent protein kinase. *Mol. Biol. Cell* **5**, 165-172.

Hoffman, P.N. and Lasek, R.J. (1975) The slow component of axonal transport: identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* **66**, 351-366.

Hoffman, P.N., Griffin, J.W. and Price, D.L.. (1984) Control of axonal caliber by neurofilament transport. *J. Cell Biol.* **99**, 705-714.

Hoffman, P.N., Griffin, J.W., Gold, B.G. and Price, D.L.. (1985) Slowing of neurofilament transport and the radial growth of developing nerve fibers. *J. Neurosci.* **5**, 2920-2929.

Hoffman, P.N., Cleveland, D.W., Griffin, J.W., Landes, P.W., Cowan, N.J. and Price, D.L. (1987) Neurofilament gene expression: a major determinant of axonal caliber. *Proc. Natl. Acad. Sci. USA* **84**, 3472-3476.

Hollenbeck, P.J. (1989) The transport and assembly of the axonal cytoskeleton. *J. Cell Biol.* **108**, 223-227.

Hollenbeck, P.J. (1996) The pattern and mechanism of mitochondrial transport in axons. *Frontiers in Bioscience (Online)* **1**, D91-102.

Hsieh, S.-T., Crawford, T.O. and Griffin, J.W. (1994a) Neurofilament distribution and organization in the myelinated axons of the peripheral nervous system. *Brain Res.* **642**, 316-326.

Hsieh, S.-T, Kidd, G.J., Crawford, T.O., Xu, Z.-S., Trapp, B.D., Cleveland, D.W. and Griffin, J.W. (1994b) Regional modulation of neurofilament organization by myelination in normal axons. *J. Neurosci.* **14**, 6392-6401.

Huber, G., Alaimo-beuret, D and Matus, A. (1985) MAP3: characterization of a novel microtubule-associated protein. *J. Cell Biol.* **100**, 496-507.

Huber, G., Pehling, G. and Matus, A. (1986) The novel microtubule-associated protein MAP3 contributes to the *in vitro* assembly of brain microtubules. *J. Biol. Chem.* **261**, 2270-2273.

Huc, C., Escurat, M., Djabali, K., Derer, M., London, F., Gros, F. and Portier, M.-M. (1989) Phosphorylation of peripherin, an intermediate filament protein, in mouse NIE 115 cell line and in sympathetic neuron. *Biochem. Biophys. Res. Commun.* **160**, 772-779.

Huxley, H.E. (1963) Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscles. *J. Mol. Biol.* **7**, 281-308.

Huxley, H.E. (1969) The mechanism of muscular contraction. *Science* **164**, 1356-1366.

Hyman, A.A., Chrétien, D, Arnal, I. and Wade, R.H. (1995) Structural changes accompanying GTP hydrolysis in microtubules: information from a slowly hydrolyzable analogue guanylyl-(α,β)-methylene-diphosphonate. *J. Cell Biol.* **128**, 117-125.

Iga, M. Inui, M. and Sobue, K. (1997) Characterization between synapsin 1 and calspectin (brain spectrin or fodrin). *Biochem. Biophys. Res. Commu.* **231**, 852-855.

Ip, W., Hartzer, M.K., Pang, Y.-Y.S. and Robson R.M. (1985a) Assembly of vimentin and its implications concerning the structure of intermediate filaments. *J. Mol. Biol.* **183**, 365-375.

Ip, W., Heuser, J.E., Pang, Y.-Y.S., Hartzer, M.K. and Robson, R.M. (1985b) Subunit structure of desmin and vimentin protofilaments and how they assemble into intermediate filaments. *Ann. New York Acad. Sci.* **455**, 185-199.

Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D. and Hartshorne, D. (1989) Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem. Biophys. Res. Commun.* **159**, 871-877.

Jacobs, M., Smith, H. and Taylor, E.W. (1974) Tubulin: nucleotide binding and enzyme activity. *J. Mol. Biol.* **89**, 455-468.

Jones, J.C.R., Goldman, A.E., Yang, H.-Y. and Goldman, R. (1985) The organizational fate of intermediate filament networks in two epithelial cell types during mitosis. *J. Cell Biol.* **100**, 93-102.

Julien, J.-P. (1995) A role for neurofilaments in the pathogenesis of amyotrophic lateral sclerosis. *Biochem. Cell Biol.* **73**, 593-597.

Julien, J.-P. and Grosveld, F. (1991) Structure and expression of neurofilament genes. *The Neuronal Cytoskeleton* (Burgoyne, R.D., editor), pp 215-231, Wiley-Liss, New-York.

Julien, J.-P. and Mushynski, W.E. (1982) Multiple phosphorylation sites in mammalian neurofilament polypeptides. *J. Biol. Chem.* **257**, 10467-10470.

Julien, J.-P. and Mushynski, W.E. (1983) The distribution of phoshorylation sites among identified proteolysis fragments of mammalian neurofilaments. *J. Biol. Chem.* **258**, 4019-4025.

Julien, J.-P., Tretjakoff, I., Beaudet, L. and Peterson. A. (1987) Expression and assembly of a human neurofilament protein in transgenic mice provide a novel neuronal marking system. *Genes and Devel.* **1**, 1085-1095.

Kanai, Y., Takemura, R., Ohshima, T., Mori, H., Ihara, Y., Yanagisawa, M., Masaki, T. and Hirokawa, N. (1989) Expression of multiple tau isoforms and microtubule bundle formation in fibroblasts transfected with a single tau cDNA. *J. Cell Biol.* **109**, 1173-1184.

Kaplan, M.P., Chin, S.S., Fliegner, K.H. and Liem, R.K.H. (1990) α-Internexin, a novel neuronal intermediate filament protein, precedes the low molecular weight neurofilament protein (NFL) in developing rat brain. *J. Neurosci.* **10**, 2735-2748.

Kaufmann, E., Geisler, N. and Weber, K. (1984) SDS-PAGE strongly overestimates the molecular masses of the neurofilament proteins. *FEBS Lett.* **170**, 81-84.

Klymkowsky, M.W. (1981) Intermediate filaments in 3T3 cells collapse after intracellular injection of a monoclonal anti-intermediate filament antibody. *Nature* **291**, 249-251.

Kotani, S., Nishida, E., Kumagai, H., and Sakai, H. (1985) Calmodulin inhibits interaction of actin with MAP2 and tau, two major microtubule-associated proteins. *J. Biol. Chem.* **260**, 10779-10783.

Krauhs, E.M., Little, M., Kempf, T., Hofer-Warbineck, R., Ade, W. and Ponstigl, H. (1981) Complete amino acid sequence of β-tubulin from porcine brain. *Proc. Natl. Acad. Sci. USA* **78**, 4156-4160.

L'Hernault, S.W. and Rosenbaum, J.L. (1985) *Chlamydomonas* α-tubulin is posttranslationally modified by acetylation of the ε-amino group of lysine. *Biochemistry* **24**, 473-478.

Lambooy, P.K. and Korn, E.D. (1988) Inhibition of an early stage of actin polymerization by actobindin. *J. Biol. Chem.* **263**, 12836-12843.

Landis, D.M.D., Hall, A.K., Weinstein, L.A. and Reese, T.S. (1988) The organization of cytoplasm at the pre-synaptic active zone of a central nervous system synapse. *Neuron* **1**, 201-209.

Lappalainen, P. and Drubin, D.G. (1997) Cofilin promotes rapid actin filament turnover *in vivo*. *Nature* **388**, 78-82.

Lasek, R.J. (1986) Polymer sliding in axons. *J. Cell Sci. Suppl.* **5**, 161-179.

Lasek, R.J., Paggi, P. and Katz, M.J. (1992) Slow axonal transport mechanisms move neurofilaments relentlessly in mouse optic axons. *J. Cell Biol.* **117**, 607-616.

Lau, S.Y.M., Taneja, A.K. and Hodges, R.S. (1984) Effect of chain length on the stabilization and formation of two-stranded α-helical coiled coils. *J. Biol. Chem.* **259**, 13253-13261.

LeDizet, M. and Pipernot, G. (1987) Identification of an acetylation site of *Chlamydomonas* α-tubulin. *Proc. Natl. Acad. Sci. USA* **84**, 5720-5724.

Lee, M.K. and Cleveland, D.W. (1996) Neuronal intermediate filaments. *Annu. Rev. Neurosci.* **19**, 187-217.

Lee, M.K., Marszalek, J.R. and Cleveland, D.W. (1994) A mutant neurofilament subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. *Neuron* **13**, 975-988.

Lee, M.K., Xu, Z, Wong, P.C. and Cleveland, D.W. (1993) Neurofilaments are obligate heteropolymers *in vivo*. *J. Cell Biol.* **122**, 1337-1350.

Lee, S., Cooper, J.A. and Pollard, T.D. (1982) Fluorescence measurement of *Acanthamoeba* profilin binding to pyrene-labeled actin. *J.Cell Biol.* **95**, 297a.

Lee, V.M.-Y., Otvos, L.Jr., Carden, M.J., Hollosi, M., Dietzschold, B. and Lazzarini, R.A. (1988) Identification of the major multiphosphorylation site in mammalian neurofilaments. *Proc. Natl. Acad. Sci. USA* **85**, 1998-2002.

Lee, V.M.-Y., Carden, M.J., Schlaepfer, W.W. and Trojanowski, J.Q. (1987) Monoclonal antibodies distinguish several differentially phosphorylated states of the two largest rat neurofilament subunits (NFH and NFM) and demonstrate their existence in the normal nervous system of adult rats. *J. Neurosci.* **7**, 3474-3488.

Lees, J.F., Shneidman, P.S., Skuntz, S.F., Carden, M.J. and Lazzarini, R.A. (1988) The structure and organization of the heavy neurofilament subunit (NFH) and the gene encoding it. *EMBO J.* **7**, 1947-1955.

Lendahl, U., Zimmerman, L. B. and McKay, D. G. (1990) CNS stem cells express a new class of intermediate filament protein. *Cell* **60,** 585-595.

Leonard, D.G.B., Gorham, J.D., Cole, P., Greene, L.A. and Ziff, E.B. (1988) A nerve growth factor-regulated messenger RNA encodes a new intermediate filament protein. *J.Cell Biol.* **106**, 181-193.

Letai, A. and Fuchs, E. (1995) The importance of intramolecular ion pairing in intermediate filaments. *Proc. Natl. Acad. Sci. USA* **92**, 92-96.

Leung, C.L. and Liem, R.K.H. (1996) Characterization of interactions between the neurofilament triplet proteins by the yeast two-hybrid system. *J. Biol. Chem.* **271**, 14041-14044.

Lewis, S.E. and Nixon, R.A. (1988) Multiple phosphorylated variants of the high molecular mass subunit of neurofilaments in axons of retinal cell neurons: characterization and evidence for their differential association with stationary and moving neurofilaments. *J. Cell Biol.* **107**, 2689-2701.

Liem R.K.H. and Hutchison, S.B. (1982) Purification of individual components of the neurofilament triplet: filament assembly from the 70 000-Dalton subunit. *Biochemistry* **21**, 3221-3226.

Liberski, P.P., Budka, H., Yanagihara, R. and Gajdusek, D.C. (1995) Neuroaxonal dystrophy in experimental Creutzfeldt-Jakob disease: electron microscopical and immunohistochemcial demonstration of neurofilament accumulations within affected neurites. *J. Comp. Path.* **112**, 243-255.

Lifsics, M.R. and Williams, R.C. (1984) Physicochemical characterization of the 68-kDa protein of bovine neurofilaments. *Biochemistry* **23**, 2866-2875.

Linck, R.W. (1989) Microtubule structure and function. *Cell Movement, Vol.2: Kinesin, Dynein, And Microtubule Dynamics* (Warner, F.D. and McIntosh, J.R., editors) pp 13-21. Alan R. Liss Inc., New-York.

Lindner, R.A. and Ralston, G.B. (1997) Macromolecular crowding: effects on actin polymerization. *Biophys. Chem.* **66**, 57-66.

Ludueña, R.F., Shooter, E.M. and Wilson, L. (1977) Structure of the tubulin dimer. *J. Biol. Chem.* **252**, 7006-7014.

Lumb, K.J. and Kim, P.S. (1995) Measurement of interhelical electrostatic interactions in the GCN4 leucine zipper. *Science*, **268**, 436-439.

Lumb, K.J., Carr, C.M. and Kim, P.S. (1994) Subdomain folding of the coiled coil leucine zipper from the bZIP transcriptional activator GCN4. *Biochemistry* **33**, 7361-7367.

MacRae, T.H. (1997) Tubulin post-translational modifications: enzymes and their mechanism of action. *Eur. J. Biochem.* **244**, 265-278.

Mandelkow, E. and Mandelkow, E.-M. (1989) Tubulin, microtubules and oligomers: molecular structure and implications for assembly. *Cell Movement, Vol.2: Kinesin, Dynein, And Microtubule Dynamics* (Warner, F.D. and McIntosh, J.R., editors) pp 23-45. Alan R. Liss Inc., New-York.

Mandelkow, E., Mandelkow, E.-M. and Milligan, R.A. (1991) Microtubule dynamics and microtubule caps: a time-resolved cryo-electron microscopy study. *J. Cell Biol.* **114**, 977-991.

Margolis, R.L. and Wilson, L. (1978) Opposite-end assembly and disassembly of microtubules at steady state *in vitro. Cell* **13**, 1-8.

Mata, M., Kupina, M. and Fink, D.J. (1992) Phosphorylation-dependent neurofilament epitopes are reduced at the node of Ranvier. *J. Neurocytol.* **21**, 199-210.

Matsumoto, G., Tsukita, S and Arai, T. (1989) Organization of the axonal cytoskeleton: differentiation of the microtubule and actin filament arrays. *Cell Movement, Vol.2: Kinesin, Dynein, And Microtubule Dynamics* (Warner, F.D. and McIntosh, J.R., editors) pp 335-356. Alan R. Liss Inc., New-York.

McNally, F.J., Okawa, K., Iwamatsu, A. and Vale, R.D. (1996) Katanin, the microtubule-severing ATPase, is concentrated at centrosomes. *J. Cell Sci.* **109**, 561-567.

Metuzals, J. and Tasaki, I. (1978) Subaxolemmal filamentous network in the giant nerve fiber of the squid (*Loligo pealei L.*) and its possible role in excitability. *J. Cell Biol.* **78**, 597-621.

Millam, L. and Erickson, H.P. (1982) Visualization of a 21 nm axial periodicity in shadowed keratin intermediate filaments. *J. Cell. Biol.* **94**, 592-597.

Mitchison, T. and Kirschner, M. (1984) Dynamic instability of microtubule growth. *Nature* **312**, 237-242.

Mitchison, T. and Kirschner, M. (1988) Cytoskeletal dynamics and nerve growth. *Neuron* **1**, 761-772.

Monteiro, M.J, Hoffman, P.N., Gearhart, J.D. and Cleveland, D.W. (1990) Expression of NFL in both neuronal and nonneuronal cells of transgenic mice: increased neurofilament density in axons without affecting caliber. *J. Cell Biol.* **111**, 1543-57.

Moritz, M., Braufeld, M.B., Sedat, J.W., Alberts, B. and Agard, D.A. (1995) Microtubule nucleation by γ-tubulin-containing rings in the centrosome. *Nature* **378**, 638-640.

Morris, J.R. and Lasek R.J. (1982) Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* **92**, 192-198.

Mulligan, L., Balin, B.J., Lee, V.M.-Y. and Ip, W. (1991) Antibody labeling of bovine neurofilaments: implications on the structure of neurofilament sidearms. *J. Struct. Biol.* **106**, 145-160.

Miyata, Y., Hoshi, M., Nishida, E., Minami, Y. and Sakai, H. (1986) Binding of microtubule-associated protein 2 and tau to the intermediate filament reassembled from neurofilament 70 KDa subunits. Its regulation by calmodulin. *J. Biol. Chem.* **261**, 13023-13030.

Myers, M.W., Lazzarini, R.A, Lee, V.M.-Y., Schlaepfer, W.W. and Nelson, D.L. (1987) The human mid-size neurofilament subunit: a repeated protein sequence and the relationship of its gene to the intermediate filament gene family. *EMBO J.* **6**, 1617-1626.

Nakagawa, T., Chen, J., Zhang, Z., Kanai, Y. and Hirokawa, N. (1995) Two distinct functions of the carboxyl terminal tail domain of NFM upon neurofilament assembly: cross-bridge formation and longitudinal elongation of filaments. *J. Cell Biol.* **129**, 411-429.

Nakamura, Y., Takeda, M., Angelides, K.J., Tanaka, T., Tada, K. and Nishimura, T. (1990) Effect of phosphorylation on 68 KDa neurofilament subunit protein assembly by the cyclic AMP dependent protein kinase *in vitro. Biochem. Biophys. Res. Commun.* **169**, 744-750.

Napolitano, E.W., Chin, S.S.M., Colman, D.R. and Liem, R.K.H. (1987) Complete amino acid sequence and *in vivo* expression of rat NF-M, the middle molecular weight neurofilament protein. *J. Neurosci.* **7**, 2590-2599.

Nash, J.A.B. and Carden, M.J. (1995) Examination of neurofilament assembly dynamics *in vitro. Biochem. Soc. Trans.* **23**, 43S.

Nautiyal, S., Woolfson, D.N., King, D.S. and Alber, T. (1995) A designed heterotrimeric coiled coil. *Biochemistry*, **34**, 11645-11651.

Nemhauser, I. and Goldberg, D.J. (1985) Structural effects of DNAseI, an actin depolymerizer that blocks fast axonal transport. *Brain Res.* **334**, 47-58.

Nishida, E, Maekawa, S. and Sakai, H. (1984a) Characterization of the action of porcine brain profilin on actin polymerization. *J. Biochem* **95**, 399-404.

Nishida, E., Maekawa, S. and Sakai, H. (1984b) Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interactions with myosin and tropomyosin. *Biochem.* **23**, 5307-5313.

Nixon, R.A. (1991) Axonal transport of cytoskeletal proteins. *The Neuronal Cytoskeleton* (Burgoyne, R.D., editor), pp 283-207, Wiley-Liss, New-York.

Nixon, R.A. and Logvinenko, K.B. (1986) Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed nonuniformly along axons of retinal ganglion cell neurons. *J. Cell Biol.* **102**, 647-659.

Nixon, R.A. and Sihag, R.K. (1991) Neurofilament phosphorylation: a new look at regulation and function. *Trends in Neurosci.* **14**, 501-506.

Nixon, R.A., Lewis, S.E., Mercken, M. and Sihag, R.K. (1994a) [^{32}P]orthophosphate and [^{35}S]methionine label separate pools of neurofilaments with markedly different axonal transport kinetics in mouse retinal ganglion cells. *Neurochem. Res.* **19**, 1445-1453.

Nixon, R.A., Paskevich, P.A., Sihag, R.K. and Thayer, C.Y. (1994b) Phosphorylation on carboxyl terminus domains of neurofilament proteins in retinal ganglion cell neurons *in vivo*: influences on regional neurofilament accumulation, interneurofilament spacing, and axon caliber. *J. Cell Biol.* **126**, 1031-1046.

Oakely, C.E. and Oakely, B.R. (1989) Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. *Nature* **338**, 662-664.

Oakely, B.R. (1992) γ-Tubulin: the microtubule organizer? *Trends Cell Biol.* **2**, 1-5.

Oblinger, M.M., Wong, J. and Parysek, L.M. (1989) Axotomy-induced changes in the expression of a type III neuronal intermediate filament gene. *J. Neurosci.* **9**, 3766-3775.

Okabe, S., Miyasaka, H. and Hirokawa, N. (1993) Dynamics of the neuronal intermediatre filaments. *J. Cell Biol.* **121**, 376-386.

Osamu, O., Gahara, Y., Miyake, T., Teraoka, H. and Kitamura, T. (1993) Neurofilament deficiency in quail caused by nonsense mutation in neurofilament-L gene. *J. Cell Biol.* **121**, 387-395.

Osborn, M. and Weber, K. (1976) Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. *Proc. Natl. Acad. Sci. USA* **73**, 867-871.

O'Shea, E.K., Klemm, J.D., Kim, P.S. and Alber, T. (1991) X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* **254**, 539-544.

O'Shea, E.K., Rutkowski, R. and Kim, P.S. (1992) Mechanism of specificity in the Fos-Jun oncoprotein heterodimer. *Cell* **68**, 699-708.

Ottaviano, Y. and Gerace, L. (1985) Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* **260**, 624-632.

Pachter J.S. and Liem R.K.H. (1984) The differential appearance of neurofilament triplet polypeptides in the developing rat optic nerve. *Developmental Biol.* **103**, 200-210.

Pant, H.C. (1988) Dephosphorylation of neurofilament proteins enhances their susceptibility to degradation by calpain. *Biochem. J.* **256**, 665-668.

Parry, D.A.D., Steven, A.C. and Steinert, P.M. (1985) The coiled-coil molecules of intermediate filaments consist of two parallel chains in exact axial register. *Biochem. Biophys. Res. Commun.* **127**, 1012-1018.

Parysek, L. M. and Goldman, R. D. (1988) Distribution of a novel 57 kDa intermediate filament (IF) protein in the nervous system, *J. Neurosci.* **8**, 555-563.

Parysek, L.M., Asnes, C.F. and Olmsted, J.B. (1984) MAP 4: occurence in mouse tissues. *J. Cell Biol.* **99**, 1309-1315.

Parysek, L.M., Chisholm, R.L., Ley, C.A. and Goldman, R.D. (1988) A type III intermediate filament gene is expressed in mature neurons. *Neuron* **1**, 395-401.

Parysek, L.M., McReynolds, M.A., Goldman, R.D. and Ley, C.A. (1991) Some neural intermediate filaments contain both peripherin and the neurofilament proteins. *J. Neurosci. Res.* **30**, 80-91.

Paschal, B.M., Shpetner, H.S. and Valee, R.B. (1987a) MAP 1C is a microtubule-activated ATPase which translocates microtubules *in vitro* and has dynein-like properties. *J. Cell Biol.* **105**, 1273-1282.

Paschal, B.M. and Valee, R.B. (1987b) Retrograde transport by the microtubule-associated protein MAP 1C. *Nature* **330**, 181-183.

Pauling, L. and Corey, R.B. (1953) Compound helical configurations of polypeptide chains: structure of proteins of the α-helical type. *Nature* **171**, 59-61.

Peter, M., Nakagawa, J., Dorée, M., Labbé, J.C. and Nigg, E.A. (1990) *In vitro* disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* **61**, 591-602.

Pettus, E.H. and Povlishock, J.T. (1996) Characterization of a distinct set of intra-axonal ultrastructural changes associated with traumatically induced alteration in axolemmal permeability. *Brain Res.* **722**, 1-11.

Pijak, D.S., Garth, F.H., Tenicki, P.J., Boulos, A.S., Lurie, D.E. and Selzer, M.E. (1996) Neurofilament spacing, phosphorylation, and axon diameter in regenerating and uninjured lamprey axons. *J. Comp. Neurol.* **368**, 569-581.

Pollard, T.D. and Cooper, J.A. (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and function. *Annu. Rev. Biochem.* **55**, 987-1035.

Pollard, T.D. and Weeds, A.G. (1984) The rate constant for ATP hydrolysis by polymerized actin. *FEBS Lett.* **170**, 94-98.

Parysek, L.M., Chrisholm, R.L., Ley, C.A. and Goldman, R.D. (1988) A type III intermediate filament gene is expressed in mature neurons. *Neuron* **1**, 395-401.

Portier, M.-M., de Néchaud, B. and Gros, F. (1984) Peripherin, a new member of the intermediate filament protein family. *Dev.Neurosci.* **6**, 335-344.

Ponstigl, H., Krauhs, E., Little, M. and Kempf, T. (1981) Complete amino-acid sequence of α-tubulin from porcine brain. *Proc. Natl. Acad. Sci. USA* **78**, 2757-2761.

Povlishock, J.T., Marmarou, A., McIntosh, T., Trojanowski, J.Q. and Moroi, J. (1997) Impact acceleration injury in the rat: evidence for focal axolemmal change and related neurofilament sidearm alteration. *J. Neuropathol. Exp. Neurol.* **56**, 347-359.

Pruss, R.M., Mirsky, R., Raff, M.C., Anderton, B. and Thorpe, R. (1980) A monoclonal antibody demonstrates that intermediate filaments share a common antigen. *J. Cell. Biol.* **94**, 178a.

Quinlan, R.A., Cohlberg, J.A., Schiller, D.L., Hatzfeld, M. and Franke, W.W. (1984) Heterotypic tetramer (A_2D_2) complexes of non-epidermal keratins isolated from cytoskeletons of rat hepatocytes and hepatoma cells. *J. Mol. Biol.* **178**, 365-388.

Quinlan, R.A., Hatzfeld, M. and Franke, W.W. (1986) Characterization of dimer subunits of intermediate filament proteins. *J.Biol.Chem.* **192** 337-349.

Riederer, B., Cohen, R. and Matys, A. (1986) MAP 5: a novel microtubule-associated protein under strong developmental regulation. *J. Neurocytol.* **15**, 763-775.

Rochlin, M.W., Wickline, K.M. and Bridgeman, P.C. (1996) Microtubule stability decreases axon elongation but not axoplasmic production. *J. Neurosci.* **16**, 3236-3248.

Rosen, D.R., Siddique, T., Patterson, D., Usdin, T.B., Sapp, P., Hetati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X. et al. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **364**, 59-62.

Rouleau, G.A., Clark, A.W., Rooke, K., Pramatarova, A., Krizus, A., Suchowersky, O., Julien, J.-P. and Figlewicz, D. (1996) SOD1 mutation is associated with accumulation of neurofilaments in amyotrophic lateral sclerosis. *Ann. Neurol.* **39**, 128-131.

Sacher, M.G., Athlan, E.S. and Mushynski, W.E. (1992) Okadaic acid induces the rapid and reversible disruption of the neurofilament network in rat dorsal root ganglion neurons. *Biochem. Biophys. Res. Commun.* **186**, 524-530.

Sacher, M.G., Athlan, E.S. and Mushynski, W.E. (1994) Increased phosphorylation of the amino-terminal domain of the low molecular weight neurofilament subunit in okadaic acid-treated neurons. *J. Biol. Chem.* **269**, 14480-14484.

Sakaguchi, T., Okada, M., Kitamura, T. and Kawasaki, K. (1993) Reduced diameter and conduction velocity of myelinated fibers in the sciatic nerve of a neurofilament deficient mutant quail. *Neurosci. Lett.* **153**, 65-68.

Sattilaro, R.F. (1986) Interaction of microtubule-associated protein 2 with actin filaments. *Biochem. J.* **25**, 2003-2009.

Schevzov, G., Gunning, P., Jeffrey, P.L., Temm Grove, C., Helfman, D.M., Lin, J.J.C. and Weinberger, R.P. (1997) Tropomyosin localization reveals distinct populations of microfilaments in neurites and growth cones. *Mol. & Cell. Neurosci.* **8**, 439-454.

Schmidt, M.L., Carden, M.J., Lee, M.M.-Y. and Trojanowski, J.Q. (1987) Phosphate dependent and independent neurofilament epitopes in the axonal swellings of patients with motor neuron disease and controls. *Lab. Invest.* **56**, 282-294.

Schnapp, B.J., Vale, R.D., Sheetz, M.P. and Reese, T.S. (1985) Single microtubules from squid axoplasm support bidirectional movement of organelles. *Cell* **40**, 455-462.

Schuermann, NFM., Hunter, J.B., Hennig, G. and Muller, R. (1991) Non-leucine residues in the leucine repeats of Fos and Jun contribute to the stability and determine the specificity of dimerization. *Nucleic Acids Res.* **19**, 739-746.

Sharp, G.A., Shaw, G. and Weber, K. (1982) Immunoelectron microscopical localization of the three neurofilament proteins along neurofilaments in cultured dorsal root ganglion cells. *Exp. Cell. Res.* **137**, 403-413.

Shaw G and Weber K (1981) Differential expression of neurofilament triplet proteins in brain development. *Nature* **298**, 277-279.

Shaw, G. (1989) Identification of previously unrecognized sequence motifs at the extreme carboxyterminus of the neurofilament subunit NFM. *Biochem. Biophys. Res. Commun.* **162**. 294-299.

Shaw, G. (1991) Neurofilament proteins. *The Neuronal Cytoskeleton* (Burgoyne, R.D., editor), pp 185-214, Wiley-Liss, New-York.

Shaw, G., Osborn, M. and Weber, K. (1981) Arrangement of neurofilaments, microtubules and microfilament-associated proteins in cultured dorsal root ganglia cells. *Eur. J. Cell Biol.* **24**, 20-27.

Schneidman, P.S., Carden, M.J., Lees, J.F. and Lazzarini, R.A. (1988) The structure of the largest murine neurofilament protein (NFH) as revealed by cDNA and genomic sequences. *Mol. Brain Res.* **4**, 217-231.

Shea, T.B., Beermann, M.L., Nixon, R.A. and Fischer, I. (1992) Microtubule-associated protein tau is required for axonal neurite elaboration by neuroblastoma cells. *J. Neurosci. Res.* **43**, 363-374.

Shiomura, Y. and Hirokawa, N (1987) The molecular structure of microtubule-associated protein 1A (MAP 1A) *in vivo* and *in vitro*. An immunoelectron microscopy and quick-freeze, deep-etch study. *J. Neurosci.* **7**, 1461-1467.

Sihag, R.K. and Nixon R.A. (1989) *In vivo* phosphorylation of distinct domains of the 70 KDa neurofilament subunit involves different protein kinases. *J. Biol. Chem.* **264**, 457-464.

Sihag, R.K. and Nixon R.A. (1990) *In vivo* phosphorylation and dephosphorylation of the 145 KDa subunit of neurofilaments: evidence for regulation by second messenger-dependent protein kinases. *J. Biol. Chem.* **265**, 4166-4171.

Sihag, R.K. and Nixon R.A. (1991) Identification of Ser^{55} as a major protein kinase A phosphorylation site on the 70-kDa subunit of neurofilaments. *J. Biol. Chem.* **266**, 18861-18867.

Skalli, O. and Goldman, R.D. (1991) Recent insights into the assembly, dynamics, and function of intermediate filament networks. *Cell Motil. Cytoskeleton* **19**, 67-79.

Sloboda, R.D., Rudolph, S.A., Rosenbaum, J.L. and Greengard, P. (1975) Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. USA* **72**, 177-181.

Small, J.V., Isenberg, G. and Gelis, J.E. (1978) Polarity of actin at the leading edge of cultured cells. *Nature* **272**, 638-639.

Spooner, B. and Holladay, C. (1981) Distribution of tubulin and actin in neurites and growth cones of differentiating nerve cells. *Cell Motil.* **1**, 167-178.

Stappenbeck, T.S., Bornslaeger, E.A., Corcoran, C.M., Luu, H.H, Virata, M.L.A. and Green, K.J. (1993) Functional analysis of desmoplakin domains: specification of the interaction with keratin versus vimentin intermediate filament networks. *J. Cell Biol.* **123**, 691-705.

Steinert, P.M. (1990) The two-chain coiled-coil molecule of native keratin intermediate filaments is a type I-type II heterodimer. *J. Biol. Chem.* **265**, 8766-8774.

Steinert, P.M. (1991a) Analysis of the mechanism of assembly of mouse keratin 1/keratin 10 intermediate filaments *in vitro* suggests that intermediate filaments are built from multiple oligomeric units rather than a unique tetrameric building block. *J. Struct. Biol.* **107**,175-188.

Steinert, P.M. (1991b) Organization of coiled-coil molecules in native mouse keratin 1/keratin 10 intermediate filaments: evidence for alternating rows of antiparallel in-register and antiparallel staggered molecules. *J. Struct. Biol.* **107**, 157-1174.

Steinert, P.M. and Liem, R.K.H. (1990) Intermediate filament dynamics. *Cell* **60**, 521-523.

Steinert, P.M. and Roop, R.R. (1988) Molecular and cellular biology of intermediate filaments. *Ann. Rev. Biochem.* **57**, 593-625.

Steinert, P.M., Marekov, L.N., Fraser, R.D. and Parry, D.A.D. (1993) Keratin intermediate filament structure: crosslinking studies yield quantitative information on molecular dimensions and mechanism of assembly. *J. Mol. Biol.* **230**, 436-452.

Sternberger, L.A. and Sternberger, N.H. (1983) Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments *in situ*. *Proc. Natl. Acad. Sci. USA.* **80**, 6126-6130.

Steven, A.C. (1990) The three dimensional structure of IFs. *Cellular and molecular biology of intermediate filaments* (Goldman, R.D. and Steinert, P.M., editors), pp 233-263, Plenum Press, New York.

Steven, A.C., Hainfeld, J.F., Trus, B.L., Wall, J.S. and Steinert, P.M. (1983) The distribution of mass in heteropolymer intermediate filaments assembled *in vitro*. *J. Biol. Chem.* **258**, 8323-8329.

Stewart, M., Quinlan, R.A. and Moir, R.D. (1989) Molecular interactions in paracrystals of a fragment corresponding to the α-helical rod portion of glial fibrillary acidic protein: evidence for an anti-parallel packing of molecules and polymorphism related to intermediate filament structure. *J. Cell Biol.* **109**, 225-234.

Straub, F.B. (1942) Actin. *Stud Szeged* **2**, 3-15.

Streifel, T.D., Avalos, R.T. and Cohlberg, J.A. (1996) cAMP-dependent phosphorylation of neurofilament protein NFL and NFM inhibits their coassembly into filaments *in vitro*. *Biochem. Biophys. Res. Commun.* **222**, 646-651.

Su, J.Y., Hodges, R.S. and Kay, C.M. (1994) Effect of chain length on the formation and stability of synthetic α-helical coiled coils. *Biochemistry* **33**, 15501-15510.

Sun, D., Macioce, P., Chin, S.S.M. and Liem, R.K.H. (1997) Assembly properties of amino- and carboxyl-terminally truncated neurofilament NFH proteins with NFL and NFM in the presence and absence of vimentin. *J. Neurochem.* **68**, 917-926.

Svitkina, T.M., Verkhovsky, A.B. and Borisy, G.G. (1996) Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. *J. Cell Biol.* **135**, 991-1007.

Takeda, S, Okabe, S. Funakoshi, T. and Hirokawa, N. (1994) Differential dynamics of neurofilament-H protein and neurofilament-L protein in neurons. *J. Cell Biol.* **127**, 173-185.

Tanaka, E., Ho, T. and Kirschner, M.W. (1995) The role of microtubule dynamics in growth cone motility and axonal growth. *J. Cell Biol.* **128**, 139-155.

Tanaka, J., Ogawara, M., Ando, S., Shibata, M., Yatani, R., Kusagawa, M. and Inagaki, M. (1993) Phosphorylation of a 62 KD porcine α-internexin, a newly identified intermediate filament protein. *Biochem. Biophys. Res. Commun.* **196**, 115-123.

Tapscott, S. J., Bennett, G. S. and Holtzer, H., Neuronal precursor cells in the chick neural tube express neurofilament proteins (1981). *Nature* **292**, 836-838.

Terada, S., Nakata, T., Peterson, A.C. and Hirokawa, N. (1996) Visualization of slow axonal transport *in vivo*. *Science* **273**, 784-788.

Thomas, G.P., Welch, W.J., Mathews, M.B. and Feramisco, J.R. (1981) Molecular and cellular effects of heat-shock and related treatments of mammalian tissue-culture cells. *Cold Spring Harbor Symp. Quant. Biol.* **46**, 985-996.

Trinkaus, J.P. (1984) *Cells into organs: the forces that shape the embryo*. pp 231-237. Prentice-Hall, New-Jersey.

Troy, C.M., Brown, K., Greene, L.A. and Shelanski, M.L. (1990a) Ontogeny of the neuronal intermediate filament protein peripherin in the mouse embryo. *Neuroscience* **36**, 217-237.

Troy, C.M., Muma, N.A., Greene, L.A., Price, D.L. and Shelanski, M.L. (1990b) Regulation of peripherin and neurofilament expression in regenerating rat motor neurons. *Brain Res.* **529**, 232-38.

Tseng, P.C.H., Runge, M.S., Cooper, J.A., Williams, R.C. and Pollard, T.D. (1984) Physical, immunochemical, and functional properties of *Acanthamoeba* profilin. J. *Cell Biol.* **98**, 214-221.

Tsukita, S. and Ishikawa, H. (1981) The cytoskeleton in myelinated axons: serial section study. *Biomed. Res.* **2**, 424-437.

Tsukita, S., Tsukita, S., Kobayashi, T. and Matsumoto, G. (1986) Subaxolemmal cytoskeleton in squid giant axon II. Morphological identification of microtubule- and microfilament- associated domains of axolemma. *J. Cell Biol.* **102**, 1710-1725.

Tu, P.-H, Elder, G., Lazzarini, R.A., Nelson, D., Trojanowski, J.Q. and Lee, V.M.-Y. (1995) Overexpression of the human NFM subunit in transgenic mice modifies the level of endogenous NFL and the phosphorylation state of NFH subunits. *J. Cell Biol.* **129**, 1629-1640.

Tu, P.-H, Raju, P., Robinson, K.A., Gurney, M.E, Trojanowski, J.Q. and Lee, V.M.-Y. (1996) Transgenic mice carrying a human mutant superoxide dismutase transgene develop neuronal cytoskeletal pathology resembling human amyotrophic lateral sclerosis lesions. *Proc. Natl. Acad. Sci. USA* **93**, 3155-3160.

Tu, P.-H., Robinson, K.A., de Snoo, F., Eyer, J., Peterson, A., Lee, V.M.-Y and Trojanowski, J.Q. (1997) Selective degeneration of Purkinje cells with Lewy body-like inclusions in aged NFHLACZ transgenic mice. *J. Neurosci.* **17**, 1064-1074.

Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W.J., Kirschner, M.W. and Cleveland, D.W. (1981) Nucleotide and corresponding amino acid sequences encoded by α- and β-tubulin mRNAs. *Nature* **289**, 650-655.

Vanderkerckhove, J. and Weber, K. (1984) Chordate muscle actins differ from invertebrate muscle actins: the evolution of the different vertebrate muscle actins. *J. Mol. Biol.* **179**, 391-413.

Vickers, J.C., Morrison, J.H., Friedrich, V.L. Jr., Elder, G.A., Perl, D.P., Katz, R.N. and Lazzarini, R.A. (1994) Age-associated and cell-type-specific neurofibrillary pathology in transgenic mice expressing the human midsized neurofilament subunit. *J. Neurosci.* **14**, 5603-5612.

Vikstrom, K.L., Borisy, G.G. and Goldman, R.D. (1989) Dynamic aspects of intermediate filament networks in BHK-21 cells. *Proc. Natl. Acad. Sci. USA* **86**, 549-553.

Vikstrom, K.L., Lim, S.-S., Goldman, R.D. and Borisy, G.G. (1992) Steady state dynamics of intermediate filament networks. *J. Cell Biol.* **118**, 1, 121-129.

Wade, R.H. and Hyman, A.A. (1997) Microtubule structure and dynamics. *Curr. Opin. Cell Biol.* **9**, 12-17.

Wegner, A. (1976) Head-to-tail polymerization of actin. *J. Mol. Biol.* **108**, 138-150.

Wegner, A. (1985) Subtleties of actin assembly. *Nature* **313**, 97-98.

Weisenberg, R.C.,Deery, W.J. and Dickinson, P.J. (1976) Tubulin-nucleotide interactions during the polymerization and depolymerization of microtubules. *Biochemistry* **15**, 4248-4254.

Welnhofer, E.A., Zhao, L. and Cohan, C.S. (1997) Actin dynamics and organization during growth cone morphogenesis in helisoma neurons. *Cell Motil. Cytoskel.* **37**, 54-71.

Wehland, J. and Weber, K. (1987) Tubulin-tyrosine ligase has a biding site on β-tubulin: a two domain structure of the enzyme. *J. Cell Biol.* **104**, 1059-1067.

Willard, M.B. and Simon, C. (1981) Antibody decoration of neurofilaments. *J. Cell Biol.* **89**, 198-205.

Wolosewick, J.J. and Porter, R. (1979) Microtrabecular lattice of the cytoplasmic ground substance (artifact or reality?). *J. Cell Biol.* **82**, 114-139.

Wong, P.C. and Cleveland, D.W. (1990) Characterization of dominant and recessive assembly-defective mutations in mouse neurofilament NFM. *J. Cell Biol.* **111**, 1987-2003.

Wong., P.C., Marszalek, J., Crawford, T.O., Xu, Z., Hsieh, S.-T., Griffin, J.W. and Cleveland, D.W. (1995) Increasing neurofilament subunit NFM expression reduces axonal NFH, inhibits radial growth, and results in neurofilamentous accumulation in motor neurons. *J. Cell Biol.* **130**, 1413-1422.

Woods, E.F. and Inglis, A.S. (1984) Organization of coiled coils in the wool microfibril. *Int. J. Biol. Macromol.* **6**, 277-283.

Xu, Z., Cork, L.C., Griffin, J.W. and Cleveland, D.W. (1993) Increased expression of neurofilament subunit NFL produces morphological alterations that resemble the pathology of human motor disease. *Cell* **73**, 23-33.

Xu, Z.-S., Liu, W.-S. and Willard, M. (1990) Identification of serine^{473} as a major phosphorylation site in the neurofilament polypeptide NFL. *J. Neurosci.* **10**, 1838-1846.

Yamasaki, H., Bennet, G.S., Itakura, C. and Mizutani, M. (1992) Defective expression of neurofilament protein subunits in hereditary hypotrophic axonopathy of quail. *Lab. Invest.* **66**, 734-743.

Yamasaki, H., Itakura, C. and Mizutani, M. (1991) Hereditary hypotrophic axonopathy with neurofilament deficiency in a mutant strain of the Japanese quail. *Acta Neuropathol.* **82**, 427-434.

Yang, Y., Dowling, J. Yu, Q.-C., Kouklis, P., Cleveland, D.W. and Fuchs, E. (1996) An essential cytoskeletal linker protein connecting actin microfilaments to intermediate filaments. *Cell* **86**, 655-665.

Yu, W. and Baas, P.W. (1995) The growth of the axon is not dependent upon the net assembly of microtubules at its distal tip. *J. Neurosci.* **15**, 6827-6833.

Zeeberg, B. and Caplow, M. (1979) Determination of free and bound microtubular protein and guanine nucleotide under equilibrium conditions. *Biochem.* **18**, 3880-3886.

Zeeberg, B., Cheek, J. and Caplow, M. (1980) Exchange of tubulin dimer into rings in microtubule assembly-disassembly. *Biochem.* **19**, 5078-5086.

Zheng, Y., Wong, M.L., Alberts, B. and Mitchison, T. (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature* **378**, 578-583.

Zimmerman, L., Parr, B., Lendahl, U., Cunningham, M. and McKay, R. (1994) Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. *Neuron* **12**, 11-24.

Zhou, N.E., Kay, C.M. and Hodges, R.S. (1994) The role of interhelical ionic interactions in controlling protein folding and stability. *J. Mol. Biol.* **237**, 500-512.

CHAPTER 2

ASSOCIATIONS BETWEEN INTERMEDIATE FILAMENT PROTEINS EXPRESSED IN CULTURED DORSAL ROOT GANGLION NEURONS

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ABSTRACT

The developmental profile of the neurofilament (NF) triplet proteins, α-internexin and peripherin in cultured dorsal root ganglion neurons from gestation day 15 rat embryos was determined by Western blot analysis. At the outset (day 0 in culture), the neurons contained mostly α-internexin. A significant increase in peripherin levels was seen at days 1-2, in the mid-sized (NFM) and low molecular weight (NFL) NF subunits at days 2-3, and in the high molecular weight (NFH) NF subunit at days 5-6. Immunofluorescence microscopy showed that the five intermediate filament proteins were colocalized in all neuronal cell bodies and neurites. Analysis of Triton X-100 extracts from okadaic acid-treated DRG cultures revealed that peripherin and α-internexin followed the same fragmentation pattern observed with NFs. Interactions between the various neuronal intermediate filament proteins in these extracts were assessed by immunoprecipitation under native conditions using antibodies specific for the individual proteins. Co-immunoprecipitation of NFH with NFL, NFM with NFL, NF-M with α-internexin, and α-internexin with peripherin demonstrated that the intermediate filament cytoskeleton in cultured sensory neurons is a highly integrated structure.

INTRODUCTION

Neurofilaments (NFs) were long considered to be the major type of intermediate filament (IF) expressed in mature neurons (Shaw et al., 1981; Trojanowski et al., 1986). However, neurons have more recently been shown to express two additional IF proteins, peripherin (Portier et al., 1984; Leonard et al., 1988; Parysek et al., 1988) and α-internexin (Fliegner et al., 1990). The expression of peripherin and α-internexin overlaps with that of NF proteins during development and in a subset of adult neurons (reviewed by Nixon and Shea, 1992).

Mammalian NFs are composed of three phosphoprotein subunits with apparent molecular masses of about 68 KDa (NFL), 150 KDa (NFM), and 200 KDa (NFH), as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Hoffman and Lasek, 1975; Julien and Mushynski, 1982). As members of the IF family of proteins, NF subunits display a characteristic domain organization, consisting of an amino-terminal head and a carboxy-terminal tail flanking a highly conserved α-helical rod domain (Geisler et al., 1983).

The occurrence of a rod domain in each NF subunit suggested that the individual proteins might be capable of forming filaments, since IF assembly begins with a coiled-coil dimerization step involving this domain (for a review see Fuchs and Weber, 1994). However, only NFL is capable of forming substantial, 10 nm-wide homopolymeric filaments *in vitro* (Geisler and Weber, 1981) and *in vivo* studies have shown that NFs are obligate heteropolymers whose formation requires NFL together with NFM and/or NFH (Ching and Liem 1993; Lee et al.,1993; Ohara et al., 1993;Nagakawa et al., 1995).

The finding that NFs are obligate heteropolymers has invited comparison with keratin IFs, which have similar properties. However, keratin IFs are stoichiometric heteropolymers requiring the participation of an acidic and a basic type keratin in coiled-coil dimerization (Fuchs and Weber, 1994). The conditions for NF assembly are less rigid, requiring NFL together with a

substoichiometric amount of either NFM or NFH (Lee et al., 1993). There is evidence that heteromeric interactions between NF subunits occur during early stages of NF assembly, at the dimer or tetramer level (Carden and Eagles, 1986; Cohlberg et al., 1995). However, the formation of NFs in cells expressing NFM or NFH at one-tenth the level of NFL (Lee et al., 1993), indicates that homopolymeric stretches can be accomodated within the NF structure.

NF assembly and structure may be more complex in neurons that also express peripherin and α-internexin. Transfection studies with an IF-deficient cell line have shown that α-internexin and peripherin can form homopolymeric filaments (Ching and Liem, 1993; Cui et al., 1995). α-Internexin can also co-assemble with each of the three NF subunits in such transfected cells (Ching and Liem, 1993) as well as *in vitro* (Balin and Miller, 1995), and peripherin was shown to co-localize with NF triplet proteins in a subset of neuronal IFs in the sciatic nerve (Parysek et al., 1991). However, little is known about the extent or nature of such interactions in neurons.

Dorsal root ganglia (DRGs) in adult rats contain two distinct types of neurons differing in size and IF content. The small neurons express peripherin, while the large neurons express NFs (Goldstein et al., 1991). On the other hand, embryonic DRGs *in vivo*, or cultures of embryonic day 15 (E15) DRG neurons contain a single neuronal type expressing both peripherin and NFs (Goldstein et al., 1996). α-Internexin is also found in embryonic DRG neurons in vivo (Fliegner et al., 1994), although its expression declines postnatally (Chiu et al., 1989; Kaplan et al., 1990).

The coassembly of different IF proteins is often assessed by methods such as immunofluorescence microscopy (Ching and Liem, 1993; Lee et al., 1993) or immunoelectron microscopy (Balin et al., 1991; Parysek et al.,1991; Balin and Miller, 1995), which provide little insight into the types of interactions involved. The availability of a method known to cause the fragmentation of NFs in cultured DRG neurons has enabled us to take a different approach to study interactions between the various neuronal IF proteins. Indeed, treating DRG

neurons with okadaic acid (OA), a potent inhibitor of protein phosphatase-2A and protein phosphatase-1 (Cohen et al., 1990) causes NFs to fragment (Sacher et al., 1992; 1994). In this report we show that peripherin and α-internexin co-localize with NFs in cultured E15 DRG neurons and undergo OA-induced fragmentation similar to that of NFs. In such OA-treated cultures, we have characterized the associations between the various neuronal IF proteins in Triton X-100 (Triton)-soluble oligomers by immunoprecipitation analysis. Our results indicate that α-internexin is a key element in the integration of the various neuronal IF proteins through its predominant association with NFM and peripherin.

MATERIALS AND METHOD

1– Materials:

OA was from LC Services (Woburn, MA). Specific, phosphorylation-independent monoclonal antibodies (Abs) against NF-L (NR4), NF-M (NN18) and NF-H (N52) were from Sigma Chemical Co. (St.Louis, MO). Monoclonal Abs against peripherin (MAB1527) and α-internexin (MAB1525) were from Chemicon International (Temecula, CA) while the one against vimentin (V9) was from Boehringer Mannheim (Montreal, Canada). Enhanced chemiluminescence reagents were from NEN (Mississauga, Canada). Neuronal IF proteins were purified by preparative SDS-PAGE as described (Julien and Mushynski, 1982). The NF proteins and α-internexin were from adult rat spinal cord while peripherin was from PC12 cells.

2– Cell Culture And Developmental Profile Neuronal IF Protein :

Rat DRGs were dissected, dispersed and maintained in defined medium as previously described (Sacher et al, 1992). For developmental analysis of neuronal IF protein expression, cells were plated on 12-well tissue culture dishes and allowed to attach to the collagen substrate for 1 hr. The medium was then drained and replaced and the first plated sample (time = 0 hr) was harvested in sample buffer (2% SDS, 62.5 mM TrisHCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol) (Laemmli, 1970). An untrypsinized sample and a trypsinized but unplated sample were also kept for analysis. Standardization of the samples was achieved by harvesting in equal volumes of sample buffer and by loading identical volumes on the gels. This is a suitable method since E15 neurons are post-mitotic and do not die to any significant degree during the first 30 to 40 days in culture (E.A., unpublished observation). For separate analysis of neurites and neuronal cell bodies, the cells that would normally be used for culture in a dispersed configuration were

concentrated down to a very small volume (10 μl), plated at the center of a 35 mm dish and allowed to attach for 0.5-1.0 hr at 37°C. The dishes were then flooded with medium and the resulting localized cultures consisted of a central neuronal cell body mass eventually surrounded by a halo of neurites.

3– OA Treatment And Time Course Analysis :

Twenty five to thirty two day-old cultures were treated with 1 μM OA for 0, 1, 2 and 4 hrs. Neuronal cell bodies and neurites were physically separated for analysis using a punch with a diameter equal to that of the cell body mass. Samples were harvested in cytoskeleton extraction buffer (CSK buffer: 1% Triton, 100 mM NaCl, 50 mM TrisHCl, pH 7.5, 50 mM NaF, 2 mM EDTA, 2 mM levamisol, 1 mM phenylmethylsulfonyl fluoride), vortexed for 30 seconds and centrifuged at 13,000x*g* for 15 minutes. The resulting pellets (13K pellet) were dissolved in SDS-Sample buffer and the 13,000x*g* supernatants (13K supernatant) were further centrifuged at 100 000x*g* in a Beckman airfuge for 5 minutes. The resulting pellets (100K pellet) and supernatants (100K supernatant) were dissolved in sample buffer or by adding one-half volume of 3x SDS-sample buffer, respectively. The 13K and 100K pellets and the 100K supernatants were in identical final volumes and equal volumes were analyzed by SDS-PAGE and Western blotting.

4– Immunoprecipitation :

13K supernatants from cultures treated with OA for 4 hrs were immunoprecipitated for 1 hr at 4°C with one of the monoclonal Abs against either NFL, NFM, NFH, α-internexin or peripherin bound to anti-mouse IgG1 Abs crosslinked to agarose beads (Sigma Chemical Co., St. Louis, MO). After centrifugation, the pelleted beads were washed repeatedly with CSK buffer. The immunoprecipitated proteins were solubilized by boiling for 5 min. in SDS sample buffer and analyzed by SDS-PAGE and Western blotting.

5– SDS-PAGE and Western blotting :

Electrophoresis of the samples on 6% SDS-polyacrylamide gels was performed as described (Laemmli, 1970). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Inc.) in a buffer containing 48 mM Tris and 39 mM Glycine. The protein bands were then detected with the Abs mentioned in the figure legends and visualized by enhanced chemiluminescence as described by the manufacturer.

6– Immunofluorescence :

Twenty-five to thirty-five day old dispersed sister cultures grown on collagen-coated glass slides were rinsed with phosphate-buffered saline (PBS: NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 10mM; KH_2PO_4, 1.8 mM; pH 7.4) and fixed with methanol for 20 min. at -20°C. Following rehydration, the samples were incubated for 30 min at 37°C with blocking buffer (PBS with 10% goat serum and 0.3% Triton) and stained for 1 hr at 37°C with a cocktail containing a rabbit polyclonal anti-NFM Ab (1:100) and one of the commercial monoclonal Abs (1:100 to 1:500) against the individual neuronal IF proteins diluted in blocking buffer. The slides were then rinsed 5 to 6 times for 5 min. with PBS containing 0.3% Triton and incubated with texas red- or DTAF-conjugated species-specific secondary Abs (1:100) and rinsed under the same conditions as used with the primary Abs. Slides were then photographed using black and white KODAK TMAX400 and a Zeiss microscope with the camera set on automatic exposure. Typical exposures were from 15 to 25 seconds for the texas-red filter and 25 to 60 seconds for the DTAF filter.

RESULTS

The specificity of the monoclonal Abs used in this study was verified by testing them individually on a Western blot containing all of the neuronal IF proteins, including phosphovariants of NFH and NFL. The results in Fig.1 indicate that the individual Abs showed no appreciable cross-reactivity with the other IF proteins. Note that since anti-α-internexin also labeled an unknown Triton-soluble band (*) migrating just above NFM, only the bottom half of Western blots was probed with this antibody.

Available *in vivo* and *in vitro* data relating to neuronal IF expression in adult and embryonic rat DRGs (Chiu et al., 1989; Kaplan et al., 1990; Fliegner et al., 1994; Goldstein et al., 1991, 1996) suggested that cultures of E15 DRG neurons would express peripherin and α-internexin along with the NF triplet proteins.The results in Fig.2 indicate that before trypsinization, E15 DRG neurons contained mainly α-internexin with traces of peripherin and NFL (Fig.2, lane B). Trypsinization alone had little impact on the profile of neuronal IF proteins (Fig.2, lane T). One hour after plating, the harvested samples had lesser amounts of these proteins, due largely to the observed failure of some neurons to attach to the collagen substrate. There was an ordered increase in the levels of neuronal IF proteins. The neurons contained mainly α-internexin at the outset (day 0). An increase in peripherin levels was seen at days 1-2, in NFL and NFM at days 2-3 and in NFH at days 5-6. Beyond day 17 the levels of all five IF proteins changed very gradually.

To address the question of whether the neuronal IF proteins co-localize in the same neurons, dispersed cultures grown on collagen-coated glass slides for twenty five days were double stained with a rabbit polyclonal anti-NFM Ab and a monoclonal Ab against one of the neuronal IF protein. The DTAF- and texas red-conjugated secondary Abs showed no detectable species cross reactivity (data not shown). The fluorescence micrographs in Fig.3 show that monoclonal Abs against the individual neuronal IF proteins each stained the

same neurites (A' to E') and neuronal cell bodies (F') as the polyclonal anti-NFM Ab (A to F), indicating that all neuronal IF proteins are completely colocalized in these cultured DRG neurons. However, differences in staining intensities of some neurites were observed, indicating that the relative proportions of different IF proteins in these processes can vary. Neuronal cell bodies were also stained by antibodies against all of the neuronal IF proteins, hence only the results for anti-NFM and anti-α-internexin are shown in Fig.3.

As a first step towards determining whether the various neuronal IF proteins are associated or form independent structures, we tested whether OA treatment would fragment assembled peripherin and α-internexin in the same way as it does NFs (Sacher et al., 1994). Localized DRG cultures were treated with 1 μM OA for 1-4 hrs, the cell bodies and neurites were harvested separately in CSK buffer and various fractions were obtained by differential centrifugation (see Materials and Methods). The 13K and 100K pellets and 100K supernatant were resolved by SDS-PAGE and Western blots were probed with Abs against NFH, NFM, NFL, α-internexin and peripherin (Fig.4). In the untreated perikaryal sample (0 hr), most of the NFH was hypophosphorylated while axonal NFH was exclusively hyperphosphorylated as can be expected from *in vivo* observations (Sternberger and Sternberger, 1983). A continuous reduction in the gel electrophoretic mobility of perikaryal NFH (dpH) was seen over the 4 hr course of OA treatment reflecting its increased phosphorylation in the presence of the phosphatase inhibitor. The perikaryal compartment also differed from the axonal compartment in that the untreated sample contained a greater proportion of Triton-soluble NFH, NFL and peripherin, perhaps reflecting the presence of newly synthesized, unassembled subunits. The amounts of all five neuronal IF proteins in the 100K pellet from cell bodies and neurites reached a maximum at 2 hrs and then levelled off. In contrast, IF protein levels in the 100K supernatant increased continuously over the 4 hr time-course, indicating that neuronal IFs in OA-treated neurons were first fragmented into large, 13,000xg-soluble oligomers and then underwent further

fragmentation to a point where they were soluble at 100,000xg. This sequence is essentially the reverse of that which occurs during incorporation of newly synthesized NF subunits into the cytoskeleton (Shea et al., 1988).

Estimates based on the Western blot in Fig.4 indicate that roughly two-thirds of the neuronal IF proteins ended up in the 13K supernatant after 4 hrs of OA treatment. A hyperphosphorylated form of NFL (pL), with a markedly reduced mobility, appeared in cell bodies and neurites at this time and was found predominantly in the 100K supernatant.

The finding that α-internexin and peripherin were also rendered Triton-soluble by OA treatment suggested that they might be coassembled with NFs. To test this possibility, Triton-soluble IF oligomers produced in DRG neurons treated with OA for 4 hrs were further analyzed by immunoprecipitation of the 13K supernatants from cell bodies and neurites with Abs against individual IF proteins. Immunoprecipitates were then analyzed by Western blotting using a cocktail of Abs against all five neuronal IF proteins as probes to determine whether any co-immunoprecipitation was taking place (Fig.5).

There are three separate controls for the immunoprecipitation data shown in Fig.5. The first (Fig.5, 50% yield) provides a 50% recovery index as it represents the IF proteins in one-half of the amount of 13K supernatant used for each immunoprecipitation. The second control (Fig.5, No Abs) shows that trace amounts of only NFM and α-internexin bound to the anti-mouse IgG1-agarose beads in the absence of an anti-IF antibody. The final control (Fig.5, 2x dilution) shows successive 2-fold dilutions of equimolar amounts of individually purified neuronal IF proteins. The strongest signal was obtained with the anti-α-internexin Ab, the weakest with anti-peripherin and anti-NFL, while Abs against NFM and NFH gave more or less equivalent signals.

The anti-NFH immunoprecipitates shown in Fig.5 contained very low amounts of the other neuronal IF proteins indicating that there was free NFH present in the extracts. However, there were large amounts of NFH in the anti-NFL immunoprecipitates, suggesting the presence of NFH/NFL

heterooligomers that either contained substoichiometric amounts of NFL or could not be immunoprecipitated by anti NFH. The next panel (anti-NFM) shows that significant amounts of NFL, α-internexin and peripherin, as well as small amounts of NFH, co-immunoprecipitated with NFM. The highest level of co-immunoprecipitation was obtained with the anti-NFL Ab, which brought down large amounts of the NF triplet proteins and lesser amounts of α-internexin and peripherin. Anti-α-internexin co-immunoprecipitated NFM and peripherin along with α-internexin. The anti-peripherin immunoprecipitates contained peripherin and α-internexin along with lower amounts of NFM. Only one-fifth of the latter immunoprecipitate was loaded on the gel due to the large amounts of peripherin in our DRG cultures and the particular effectiveness of the anti-peripherin Ab for immunoprecipitation.

To further confirm that co-immunoprecipitation of neuronal IF proteins was not due to the fortuitous association of IF proteins in general, the Western blots were stripped and reprobed with an anti-vimentin Ab. The latter showed that there was virtually no vimentin in any of the immunoprecipitates although an appreciable amount could be seen in the initial Triton-soluble extracts (Fig.5 bottom panel).

DISCUSSION

In this report, we show that cultured E15 DRG neurons from rat embryos resemble their *in vivo* counterparts in certain aspects of their developmental expression of neuronal IF proteins. Examination of Fig.2 indicated that α-internexin was the predominant species in freshly dissected DRGs and for the first 2 days after plating, consistent with its early expression *in vivo* (Fliegner et al., 1994). The early increase in peripherin levels contradicted reports of its tightly coordinated expression with NFL in DRGs (Escurat et al., 1990). On the other hand, the observed coexpression of NFL and NFM concurs with the results of previous studies (Carden et al., 1987), as does the delay in onset of NFH expression (Shaw and Weber, 1982; Pachter and Liem, 1984; Lindenbaum et al., 1988).

As reported previously (Goldstein et al., 1996), E15 DRG neurons fail to differentiate *in vitro* into two distinct phenotypes expressing either peripherin or NFs. In addition to coexpression of peripherin and NF proteins (Goldstein et al., 1996), we have shown that they retain another embryonic feature, the continued expression of α-internexin. Thus, the reciprocal changes in the levels of NFL and α-internexin expression that occur during development (Fliegner et al., 1990) were not seen. These discrepancies may be due to the inability of cultured neurons to establish contact with appropriate target cells as attempts to normalize IF protein expression in DRG neurons through the addition of skeletal and heart muscle extracts has met with limited success (Goldstein et al., 1996).

Following the fragmentation pattern of the neuronal IF network in OA-treated cultures provided some insight into the relationship between the different components. In addition to NF proteins, the Triton solubility of peripherin and α-internexin also increased under these conditions. More significantly, the time-course and extent of solubilization was the same for all of

these proteins in both the perikaryal and axonal compartments, suggesting that they were interconnected.

OA-induced NF fragmentation is reversible to a point (Sacher et al., 1992) and is likely due to inhibition of protein phosphatase-2A (Cohen et al., 1990; Sacher et al., 1994), an enzyme associated with NFs that has been proposed to play a role in preserving their filamentous structure (Saito et al., 1995). Moreover, NF fragmentation correlates with an increase in the phosphorylation of two protein kinase A sites in the head domain of NFL (Giasson et al., 1996). Head domain phosphorylation may similarly affect α-internexin (Tanaka et al., 1993), but there is a lack of evidence linking peripherin phosphorylation with disassembly (Aletta et al., 1989). Nevertheless, it appears that OA shifts the equilibrium between the antagonistic effects of protein phosphatase-2A and protein kinase A (Giasson et al., 1996), apparently magnifying an oligomerization process involved in NF dynamics (Okabe et al., 1993). Thus, IF proteins contained in the oligomeric products of OA-induced disassembly are likely to retain the normal association patterns that exist *in situ*.

The validity of the co-immunoprecipitation paradigm we used to test for possible associations between the various neuronal IF proteins is based upon several criteria. Firstly, the Western blot in Fig.1 indicated that the individual monoclonal Abs we used did not show any cross reactivity with other IF proteins. Secondly, the control in Fig.5 carried out in the absence of primary Abs showed that the fragmented IFs remained soluble throughout the immunoprecipitation protocol. Furthermore, reference to the 50% yield index in Fig.5 indicated that in some cases, co-immunoprecipitated species comprised up to half of the starting material and were likely to be representative of the total fraction. Also noteworthy was the lack of vimentin, a component of satellite cells in DRG cultures, in the immunoprecipitates. All three NF subunits have been shown to coassemble with vimentin in co-transfected non-neuronal cells (Chin and Liem., 1989, 1990; Monteiro and Cleveland, 1989). The failure of vimentin

to co-immunoprecipitate with any of the neuronal IF proteins indicated that the associations we observed did not result from interactions taking place in the Triton extracts following cell lysis.

The co-immunoprecipitation data showed several associations between neuronal IF proteins, but certain discrepancies were apparent. The main problem relates to the apparent lack of reciprocity between co-immunoprecipitates obtained with anti-NFL as compared to those obtained with anti-NFH, anti-α-internexin and anti-peripherin, respectively. A high level of co-immunoprecipitation of all the neuronal IF proteins was effected by anti-NFL, perhaps reflecting its key role in NF assembly (Ohara et al., 1993). On the other hand, only low amounts of NFL were co-immunoprecipitated by anti-NFH, and none was seen in the anti-α-internexin and anti-peripherin immuno-precipitates. These apparent discrepancies can be explained in several ways. In the case of NFH, it is possible that two forms of this subunit are present in the Triton-soluble fraction, one consisting of free NFH and the other comprising NFH associated with NFL in a form that is refractory to immunoprecipitation by anti-NFH. It is also possible that NFH is associated with heterooligomers containing substoichiometric amounts of NFL. However, the latter possibility cannot apply to the anti-α-internexin and anti-peripherin immunoprecipitates because they did not contain NFL. In these two cases it appears that epitope masking may take place in those heterooligomers that contain NFL along with α-internexin and/or peripherin. Although the basis for these discrepancies remains to be determined, they do not detract from the validity of our data in demonstrating associations between the various neuronal IF proteins.

The low level of association between NFM and NFH indicated by the co-immunoprecipitation studies suggests that the two subunits may be contained in different subsets of oligomers. This difference may reflect a fundamental aspect of NF organization and could explain the more dynamic nature of NFH in neurons (Takeda et al., 1994).

The present study provides direct evidence for an association between α-internexin, NFM and peripherin in cultured E15 DRG neurons, indicating that the IF cytoskeleton in these cells is a highly integrated structure. Perhaps the coassembly of α-internexin and peripherin with NFs produces IFs with a high degree of plasticity required to support neurite outgrowth (Nixon and Shea, 1992). This notion concurs with the presence of high levels of α-internexin in embryonic DRGs (Fliegner et al., 1994) and with the upregulation of peripherin in large NF-containing DRG neurons during axonal regeneration (Oblinger et al., 1989). The elucidation of these and other questions pertaining to the neuronal IF cytoskeleton will no doubt be facilitated by the availability of cultured neurons that coexpress all of the major neuronal IF proteins.

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REFERENCES

Aletta, J.M., Shelanski, M.L. and Greene, L.A. (1989) Phosphorylation of the peripherin 58 kDa neuronal intermediate filament protein, Regulation by nerve growth factor and other agents. *J. Biol. Chem.* **264**, 4619-4627.

Balin, B.J. and Lee, V.M.-Y. (1991) Individual neurofilament subunits reassembled *in vitro* exhibit unique biochemical morphological and immunological properties. *Brain Res.* **556**, 196-208.

Balin, B.J. and Miller, M.E. (1995) Reassembly of the 66 kD neurofilament protein in vitro following isolation and purification from bovine spinal cord. *J. Neurosci. Res.* **40**, 79-88.

Carden, M.J. and Eagles, P.A.M. (1986) Chemical cross-linking analyses of ox neurofilaments. *Biochem. J.* **234**, 587-591.

Carden, M.J., Trojanowski, J.Q., Schlaepfer, W.W. and Lee, V.M.-Y. (1987) Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J Neurosci.* **7**, 3489-3504.

Chin, S.S.M. and Liem, R.K.H. (1989) Expression of rat neurofilament proteins NFL and NFM in transfected non-neuronal cells. *Eur. J. Cell Biol.* **50**, 475-490.

Chin, S.S.M, and Liem, R.K.H. (1990) Transfected rat high-molecular-weight neurofilament (NFH) coassembles with vimentin in a predominantly nonphosphorylated form. *J Neurosci.* **10**, 3714-3726.

Ching, G.Y. and Liem, R.K.H. (1993) Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. *J. Cell Biol.* **122**, 1323-1335.

Chiu, F.C., Barnes, E.A., Das, K., Haley, J., Socolow, P., Macaluso, F.P. and Fant J. (1989) Characterization of a novel 66 Kd subunit of mammalian neurofilaments. *Neuron* **2**, 1435-1445.

Cohen, P., Holmes, C.F.B., and Tsukitani Y. (1990) Okadaic acid: A new probe for the study of cellular regulation. *Trends Biochem. Sci.* **15**, 98-102.

Cohlberg, J.A., Hajarian, H., Tran, T., Alipourjeddi P., and Noveen A. (1995) Neurofilament protein heterotetramers as assembly intermediates. *J. Biol. Chem.* **270**, 9334-9339.

Cui, C., Stambrook, P.J. and Parysek, L.M. (1995) Peripherin assembles into homopolymers in SW13 cells. *J. Cell Sci.* **108**, 3279-3284.

Escurat, M., Djabali, K., Gumpel, M., Gros, F. and Portier, M.-M. (1990) Differential expression of two neuronal intermediate filament proteins peripherin and the low molecular mass neurofilament protein (NF-L) during the development of the rat. *J. Neurosci.* **10**, 764-784.

Fliegner, K.H., Ching, G.Y. and Liem, R.K.H. (1990) The predicted amino acid sequence of α-Internexin is that of a novel neuronal intermediate filament protein. *EMBO J* **9**, 749-755.

Fliegner, K.H., Kaplan, M.P., Wood, T.L., Pintar, J.E. and Liem, R.K.H. (1994) Expression of the gene for the neuronal intermediate filament protein α-internexin coincides with the onset of neuronal differentiation in the developing rat nervous system. *J. Comp. Neurol.* **342**, 161-173.

Fuchs, E. and Weber, K. (1994) Intermediate filaments: structure dynamics function and disease. *Annu. Rev. Biochem.* **63**, 345-382.

Geisler, N. and Weber, K. (1981) Self-assembly in vitro of the 68000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate sized filaments. *J. Mol. Biol.* **151**, 565-571.

Geisler, N., Kaufmann, E., Fischer, S., Plessman, U. and Weber. K. (1983) Neurofilament architecture combines structural principles of intermediate filaments with C-terminal extensions increasing in size between triplet proteins. *EMBO J.* **2**, 1295-1302.

Giasson, B.I., Cromlish, J.A., Athlan, E.S. and Mushynski, W.E. (1996) Activation of cyclic AMP-dependent protein kinase in okadaic acid-treated neurons potentiates neurofilament fragmentation and stimulates phosphorylation of Ser-2 in the low-molecular-mass neurofilament subunit. *J. Neurochem.* **66**, 1207-1213.

Goldstein, M.E., House, S.B. and Gainer, H. (1991) NFL and peripherin immunoreactivities define distinct classes of rat sensory ganglion cells. *J. Neurosci. Res.* **30**, 92-104.

Golstein, M.E., Grant, P., House, S.B., Henken, D.B. and Gainer, H. (1996) Developmental regulation of two distinct neuronal phenotypes in rat dorsal root ganglia. *Neuroscience* **71**, 243-258.

Harris, J., Ayyub, C. and Shaw, G. (1991) A molecular dissection of the carboxy-terminal tails of the major neurofilament subunits NFM and NFH. *J. Neurosci. Res.* **30**, 47-62.

Hoffman, P.N. and Lasek, R.J. (1975) The slow component of axonal transport: Identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* **66**, 351-366.

Julien, J.-P., Mushynski, W.E. (1982) Multiple phosphorylation sites in mammalian neurofilament polypeptides. *J. Biol Chem.* **257**, 10467-10470.

Kaplan, M.P., Chin, S.S.M., Fliegner, K.H. and Liem, R.K.H. (1990) α-Internexin, a novel neuronal intermediate filament protein precedes the low molecular weight neurofilament protein (NFL) in the developing rat brain. *J. Neurosci.* **10**, 2735-2748.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, M.K., Xu, Z., Wong, P.C. and Cleveland, D.W. (1993) Neurofilaments are obligate heteropolymers *in vivo*. *J. Cell Biol.* **122**, 1337-1350.

Leonard, D.G.B., Gorham, J.D., Cole, P., Greene, L.A. and Ziff, E.B. (1988) A nerve growth factor-regulated messenger RNA encodes a new intermediate filament protein. *J. Cell Biol.* **106**, 181-193.

Lindenbaum, M.H., Carbonetto, S., Grosveld, F., Flavell, D., and Mushynski, W.E. (1988) Transcriptional and post-transcriptional effects of nerve growth factor on expression of the three neurofilament subunits in PC12 cells. *J. Biol. Chem.* **263**, 5662-5667.

Monteiro, M.J. and Cleveland, D.W. (1989) Expression of NFL and NFM in fibroblasts reveals coassembly of neurofilament and vimentin subunits. *J. Cell Biol.* **108**, 579-593.

Nagakawa, T., Chen, J., Zhang, Z., Kanai, Y. and Hirokawa, N. (1995) Two distinct functions of the carboxy terminal tail domain of NFM upon neurofilament assembly: crossbridge formation and longitudinal elongation of filaments. *J. Cell Biol.* **129**, 411-429.

Nixon, R.A. and Shea, T.B. (1992) Dynamics of neuronal intermediate filaments: a developmental perspective. *Cell Motil. Cytoskeleton* **22**, 81-91.

Oblinger, M.M., Wong, J. and Parysek, L.M. (1989) Axotomy-induced changes in the expression of a type III neuronal intermediate filament gene. *J. Neurosci.* **9**, 3766-3775.

Ohara, O., Gahara, Y., Miyake, T., Teraoka, H. and Kitamura, T. (1993) Neurofilament deficiency in quail caused by nonsense mutation in neurofilament-L gene. *J. Cell Biol.* **121**, 387-395.

Okabe, S., Miyasaka, H. and Hirokawa, N. (1993) Dynamics of the neuronal intermediate filaments. *J. Cell Biol.* **121**, 375-386.

Pachter, J.S. and Liem, R.K.H. (1984) The differential appearance of neurofilament triplet polypeptides in the developing rat optic nerve. *Developmental Biol.* **103**, 200-210.

Parysek, L.M., Chrisholm, R.L., Ley, C.A. and Goldman, R.D. (1988) A type III intermediate filament gene is expressed in mature neurons. *Neuron* **1**, 395-401.

Parysek L.M., McReynolds, M.A., Goldman, R.D. and Ley, C.A. (1991) Some neural intermediate filaments contain both peripherin and the neurofilament proteins. *J. Neurosci. Res.* **30**, 80-91.

Portier, M.-M., de Néchaud, B., Gros. F. (1984) Peripherin, a new member of the intermediate filament protein family. *Dev. Neurosci.* **6**, 335-344.

Sacher, M.G., Athlan, E.S. and Mushynski, W.E. (1992) Okadaic acid induces the rapid and reversible disruption of the neurofilament network in rat dorsal root ganglion neurons. *Biochem. Biophys. Res. Commun.* **186**, 524-530.

Sacher, M.G., Athlan, E.S. and Mushynski, W.E. (1994) Increased phosphorylation of the amino-terminal domain of the low molecular weight neurofilament subunit in okadaic acid-treated neurons. *J. Biol. Chem.* **269**, 18480-18484.

Saito, T., Shima, H., Osawa, Y., Nagao, M., Hemmings, B.A., Kishimoto, T. and Hisanaga, S.I. (1995) Neurofilament-associated protein phosphatase 2A: Its possible role in preserving neurofilaments in filamentous states. *Biochemistry* **34**, 7376-7384.

Shaw, G., Weber, K. (1981) Differential expression of neurofilament triplet proteins in brain development. *Nature* **298**, 277-279.

Shaw, G., Osborn, M, and Weber, K. (1982) An immunofluorescence microscopical study of the neurofilament triplet proteins vimentin and glial fibrillary acidic protein in the adult rat brain. *Eur. J. Cell Biol.* **26**, 68-82.

Shea, T.B., Sihag, R.K. and Nixon, R.A. (1988) Neurofilament triplet proteins of NB2a/dl neuroblastoma: posttranslational modification and incorporation into the cytoskeletion during differentiation. *Dev. Brain Res.* **43**, 97-109.

Sternberger, L.A. and Sternberger, N.H. (1983) Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. *Proc. Natl. Acad. Sci. USA* **80**, 6126-6130.

Takeda, S., Okabe, S., Funakoshi, T. and Hirokawa, N. (1994) Differential dynamics of neurofilament-H protein and neurofilament-L protein in neurons. *J. Cell Biol.* **127**, 173-185.

Tanaka, J., Ogawara, M., Ando, S., Shibata, M., Yatani, R., Kusagawa, M., Inagaki, M. (1993) Phosphorylation of 62 kDa porcine α-internexin, a newly identified intermediate filament protein. *Biochem. Biophys. Res. Commun.* **196**, 115-123.

Trojanowski, J.Q., Walkenstein, N. and Lee, V.M.-Y. (1986) Expression of neurofilament subunits in neurons of the central and peripheral nervous system: An immunohistochemical study with monoclonal antibodies. *J. Neurosci.* **6**, 650-660.

Figure 1: Verification of the specificity of the monoclonal Abs against the individual neuronal IF proteins by Western blot analysis. DRG samples containing the various phosphovariants of NFL and NFH were resolved by SDS-PAGE and individual lanes were developed with one of the monoclonal Abs. The letters H, M, L, I and P at the top of the individual lanes refer to the monoclonal Abs against NFH, NFM, NFL, α-internexin and peripherin, respectively. pH and dpH, hyper- and hypophosphorylated forms of NF-H, respectively; M, NF-M; pL and L, hyperphosphorylated and normal NF-L, respectively; I, α-internexin; P, Peripherin; *, a cross reactive band of unknown origin.

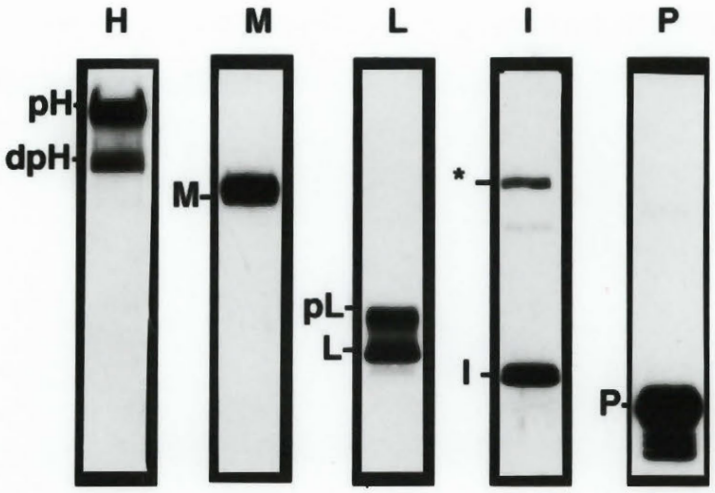

H
M
L
I
P
pH
dpH
M
pL
L
*
I
P

Figure 2: Western blot analysis of the developmental profile of neuronal IF proteins in cultured E15 DRG neurons. DRGs were cultured as described in Materials and Methods and harvested in equal volumes of sample buffer at the times (in days) indicated at the top of each lane. In the panel on the right, sample volumes were one-fourth of those in the left-hand panel to permit visualization of the entire range of samples with a single film exposure. The day 10 sample was reloaded in the right panel at one-fourth the volume for comparison. The upper part of the blot was probed with monoclonal Abs against NFH and NFM while the lower part was probed with monoclonal Abs against NFL, α-internexin and peripherin. IF protein bands are designated as described in the legend to Fig.1.

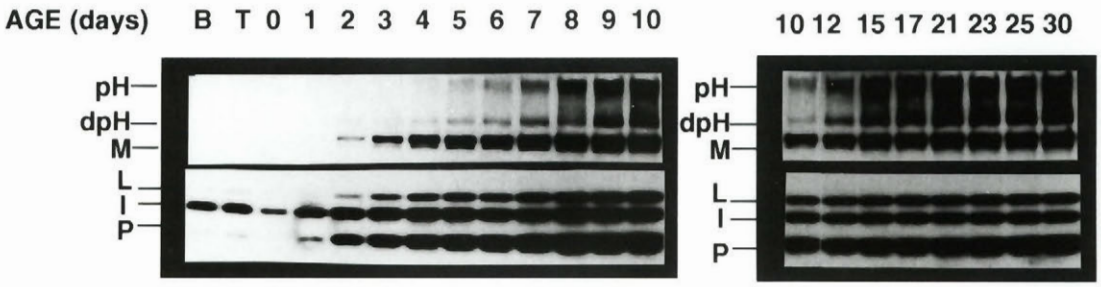
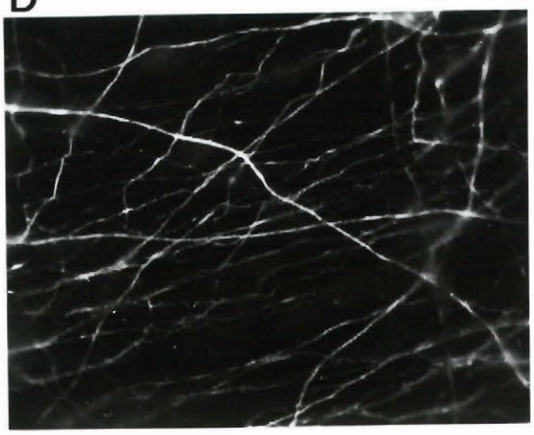
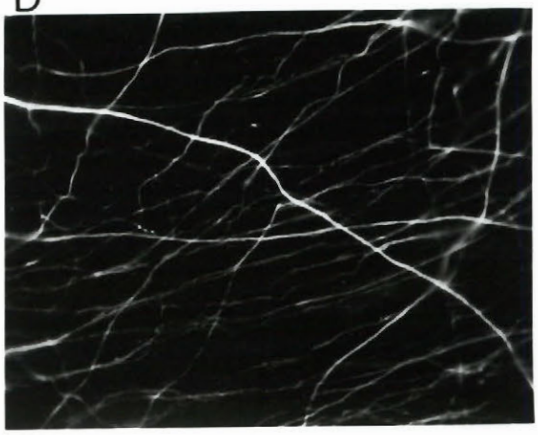

AGE (days)
B T 0 1 2 3 4 5 6 7 8 9 10
10 12 15 17 21 23 25 30
pH
dpH
M
L
I
P

Figure 3: Co-localization of neuronal IF proteins in cultured E15 DRG neurons by immunofluorescence microscopy. Cells were plated on collagen-coated glass slides, fixed and stained as described in Materials and Methods. Panels A to F: sister cultures stained with a rabbit polyclonal anti-NFM Ab. Panels A' to F': co-staining with monoclonal Abs specific for NFH (A'), NFM (B'), NFL (C'), peripherin (D') and α-internexin (E' and F'). Panels A, A' to E, E' show staining of neurites while F and F' show cell body staining.

D

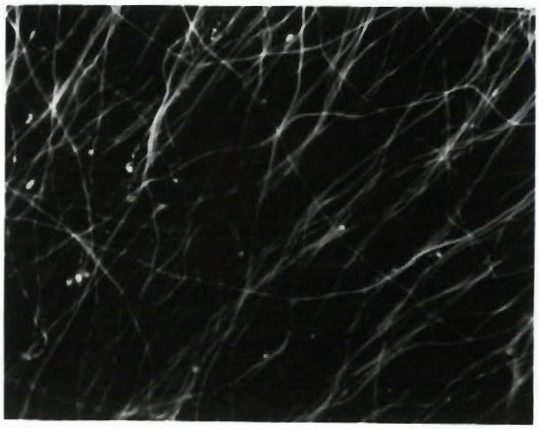


D'

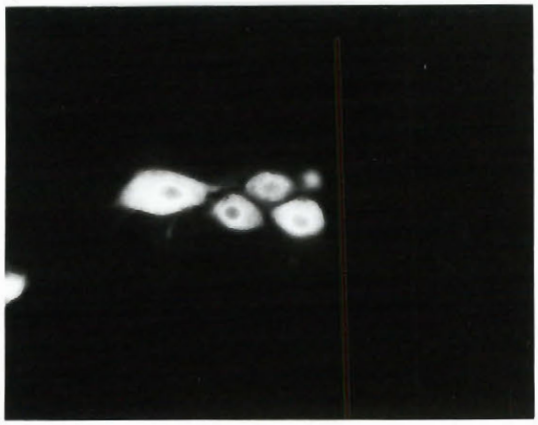


E

E'



F



F'

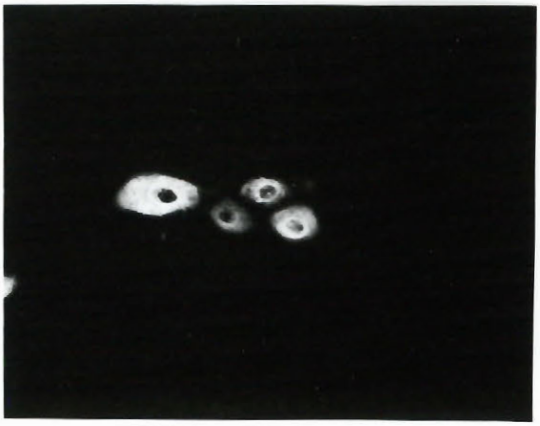


Figure 4: Western blot analysis of the time course of neuronal IF fragmentation in OA-treated neurons. Localized DRG cultures were treated with 1 μM OA for 0, 1, 2 or 4 hrs. The cell bodies and neurites were separately harvested in CSK buffer and centrifuged at 13,000 xg for 15 min. The pellets (13K pellet) were dissolved in SDS-sample buffer, and the supernatants were centrifuged at 100,000 xg for 5 min. yielding 100,000 xg-insoluble (100K pellet) and -soluble (100K super) fractions. Pellets and supernatants were resolved by SDS-PAGE and analyzed by Western blotting as described in the legend to Fig.2. IF protein bands are designated as described in the legend to Fig.1.

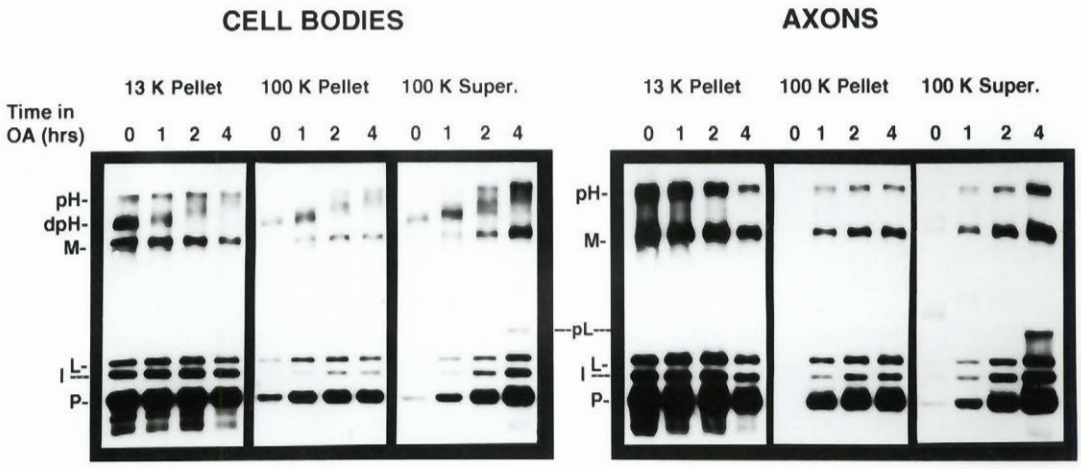
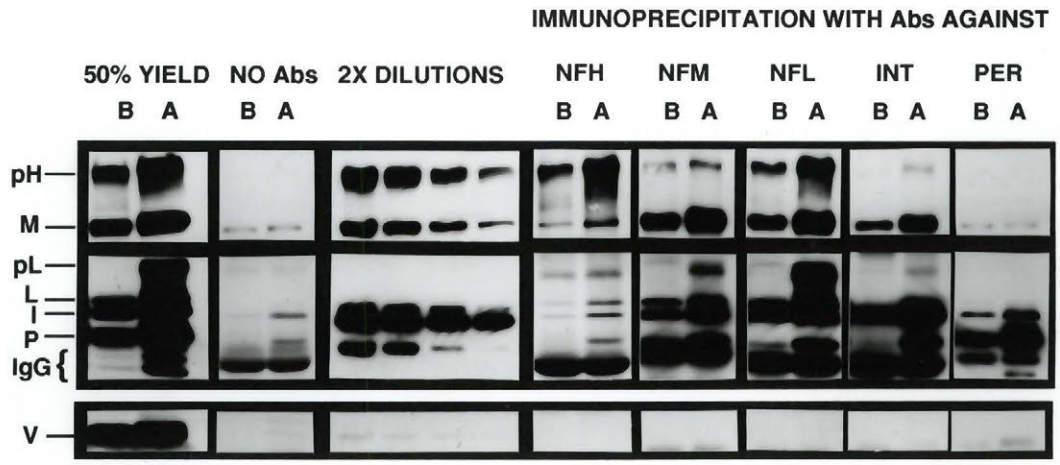

CELL BODIES
AXONS
13 K Pellet
100 K Pellet
100 K Super.
Time in OA (hrs)
0 1 2 4
pH-
dpH-
M-
pL
L-
P-

Figure 5: Western blot analysis of immunoprecipitates from the 13,000 xg Triton-soluble fractions of cell bodies and neurites of OA-treated neurons. Localized DRG cultures were treated with 1 μM OA for 4 hrs; the cell bodies and neurites were separately harvested in CSK buffer and centrifuged at 13,000 xg for 15 min. The supernatants were immunoprecipitated as described in materials and methods. The panel designations at the top include: 50% YIELD: each lane contains one-half of the amount of 13K supernatant fraction used for immunoprecipitation; NO Abs: control with no immunoprecipitating antibody. 2x DILUTIONS: successive 2-fold dilutions of equimolar amounts of all 5 neuronal IF proteins. NFH, NFM, NFL, INT and PER refer to immunoprecipitates obtained with anti-NFH, anti-NFM, anti-NFL, anti-α-internexin and anti-peripherin, respectively. B and A refer to the cell body and neurite fractions, respectively. Western blots were probed as described in the legend to fig.2. IF protein bands are designated as described in the legend to Fig.1. The major band just under peripherin is the IgG heavy chain (IgG).


IMMUNOPRECIPITATION WITH Abs AGAINST
50% YIELD
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NFH
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CHAPTER 3

HETERODIMERIC ASSOCIATIONS BETWEEN NEURONAL INTERMEDIATE FILAMENT PROTEINS

Eric S. Athlan and Walter E. Mushynski

ABSTRACT

Formation of protein dimers involving α-internexin, peripherin and the neurofilament proteins, NFH, NFM and NFL, were investigated by partial renaturation of various combinations of individually purified subunits in buffered 2 M urea. Oligomers that were formed were resolved by "blue" native electrophoresis (Schägger, H., Cramer, W.A., and von Jagow, G. (1994) *Anal. Biochem.* **217**, 220-230) modified to include urea in the polyacrylamide gels. Combining this method with Western blot analysis, disulfide cross-linking and SDS-polyacrylamide gel electrophoresis in the second dimension showed that NFL readily forms significant amounts of heterodimer with NFH, NFM, α-internexin or peripherin in the presence of 2 M urea. α-Internexin and peripherin also formed heterodimers with NFH or NFM under these conditions. The modified version of "blue" native gel electrophoresis described here may be useful in monitoring the impact of post-translational modifications and mutations on the dimerization of intermediate filament proteins.

INTRODUCTION

The cytoskeleton of most eukaryotic cells includes an intermediate filament (IF)[1] network assembled from protein subunits expressed in a cell specific manner (see Fuchs and Weber, 1994, for a review). IF proteins belong to a large family of related polypeptides that share a characteristic tripartite domain organization consisting of a conserved, ~310 amino acid-long, α-helical "rod" domain flanked by amino-terminal "head" and carboxy terminal "tail" domains of more heterogeneous length and composition (see Steinert and Roop, 1988, for a review). The first step in IF assembly involves dimerization of two identical subunits, as seen with vimentin and desmin (Quinlan et al., 1986), or two different subunits in the case of keratins (Hatzfeld et al., 1990; Steinert, 1990). This initial dimerization step produces an unstaggered, parallel, coiled-coil species (Fuchs and Weber, 1994) that can be chemically cross-linked with the copper phenanthroline (CuP_2) reagent in cases where the subunits contain a cysteine residue located in the rod domain (Quinlan et al., 1986).

Neuronal cells from cultured embryonic rat dorsal root ganglia (DRGs) are unusual in that they contain at least five different developmentally regulated and coassembled neuronal IF (nIF) subunits (Athlan et al., 1997). They include α-internexin (Chiu et al., 1989), peripherin (Portier et al., 1984) and the neurofilament (NF) triplet proteins, which consist of the heavy (NFH), mid-sized (NFM) and low molecular weight (NFL) subunits (Hoffman and Lasek, 1975). Among these five nIF proteins, only NFL has been purified and chemically cross-linked as a dimer (Quinlan et al., 1986), although disulfide-linked peripherin dimers that occur naturally *in vivo* have been reported (Chadan et al., 1994). NFs appear to be obligate heteropolymers *in vivo* (Lee et al., 1993; Ching and Liem, 1993) and individual NF subunits can also be incorporated into an α-internexin (Ching and Liem, 1993), peripherin (Parysek et al., 1991) or vimentin (Chin and Liem, 1989; Sun et al., 1997) network.

Several lines of evidence support or suggest the existence of NFH/NFL or NFM/NFL heterodimers as the smallest heteromeric unit of NF assembly. For instance, cross-linking analysis of native or partially disassembled NFs revealed that NFM could be cross-linked to NFL through cysteine residues located in the rod domain, although the approach that was used could not differentiate intra- from inter-coiled-coil dimer cross-linking (Carden and Eagles, 1986). Furthermore, Mulligan *et al.* (1991) concluded that the existence of NFH/NFL and NFM/NFL heterodimers could best reconcile their immunoelectron microscopic data with the available literature (see discussion in Mulligan et al.,1991). Finally, NFH and NFM were found to interact more strongly with NFL than with themselves or with each other in a yeast two hybrid system paradigm, suggesting that heterodimer formation with NFL is favored over NFH and/or NFM oligomerization (Carpenter and Ip., 1996; Leung and Liem, 1996).

To date, however, the smallest NF hetero-oligomers examined biochemically were reported to be heterotetramers containing equimolar amounts of NFH or NFM with NFL. However, it could not be determined whether these were assembled from individual homo- or hetero- dimers (Cohlberg et al., 1995). Moreover, Triton-soluble heterooligomers of α-internexin, peripherin and the NF subunits have been reported, but their precise composition also remains unknown (Athlan et al., 1996). Thus, the precise nature of the smallest heterooligomers involving the various nIF proteins is still elusive, due largely to the limitations of experimental approaches capable of resolving and characterizing these small *in vivo* or *in vitro* assembly intermediates.

A novel "blue" native polyacrylamide gel electrophoresis (BN-PAGE) system has recently been described in which Coomassie Blue G-250 is used to confer a negative charge to protein complexes, allowing them to be separated on the basis of their molecular mass while preserving their native associations (Schägger et al., 1994). Initial studies conducted in our laboratory showed that BN-PAGE, rendered "semi-native" by inclusion of 2 M urea,

resolved nIF proteins into what appeared to be heterocomplexes. Further studies of nIF protein complexes were initiated in which various combinations of individually purified, denatured subunits were assembled by dialysis against buffered 2 M urea. Subsequent analysis of assembled protein complexes by BN-PAGE/2 M urea, together with other techniques, demonstrated that NFL forms heterodimers with NFH or NFM and that α-internexin and peripherin can form heterodimers with the individual NF subunits. These results indicate that previously reported interactions between the various nIF proteins (Athlan et al., 1997) may take place at the level of heterodimer formation.

MATERIALS AND METHOD

1– Preparation of Cytoskeletal Extracts:

13000 x g-insoluble cytoskeletal extracts of embryonic cultured DRG neurons and PC12 cells were prepared essentially as described (Athlan et al., 1996).

2– Purification of Neuronal Intermediate Filament Proteins:

NFH, NFM, NFL and α-internexin were isolated as NFs from rat spinal cord as described (Julien and Mushynski, 1982) while peripherin was from Triton X-100-insoluble PC-12 cell extracts. These preparations were solubilized and boiled for 5 min in SDS-sample buffer (2% SDS, 62.5 mM Tris (pH 6.8), 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol) (Laemli, 1970) and separated by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 15 mM thioglycolic acid to prevent oxidation of the cysteine residues. The proteins of interest were visualized, excised and electro-eluted as previously described (Julien and Mushynski, 1982). The electro-eluted material was recovered and solid urea was added to a concentration of 2M. These SDS-containing solutions were then applied to individual columns of "Extractigel" detergent-removing matrix (Pierce) equilibrated with a degassed buffer containing 2M Urea, 50 mM Tris (pH 9.0), 5 mM DTT. Following elution with the same buffer, an aliquot was removed for quantification and verification of protein purity and for SDS assay to ensure that the detergent had been removed (Sokoloff and Frigon, 1981). The protein solutions were dialyzed against a buffer containing 6 M guanidine hydrochloride (gua-HCl), 50 mM Bis Tris (pH 7.0), 5 mM DTT by repeated centrifugations in a "Centricon" dialysis spin column (Amicon, Montreal, Canada), aliquoted and stored at -80°C until further use. Proteins quantified by the bicinchoninic acid method (Sigma) were purified in the absence of reducing agents and the SDS removal protocol was replaced with dialysis against 5mM

NaCl, 0.01% SDS, in the Centricon spin columns. This permitted the removal of Tris, glycine, DTT and excess SDS, which may interfere with the protein assay.

3– Cell Culture :

Embryonic rat DRGs were dissected, dispersed and maintained in defined medium as previously described (Athlan et al., 1996; Giasson and Mushynski, 1996).

4– Partial Assembly of Neuronal IF Proteins :

The individually purified nIF proteins were mixed (final concentration 0.1 mM to 1.0 mM) and dialyzed in a Microdialyzer apparatus (Pierce) at room temperature for 2.5 hrs against 2-3 changes of an extensively degassed buffer containing 50 mM Bis Tris (pH 7.0), and urea at the concentration specified in the figure legends.

5– Disulfide Cross Linking of Neuronal IF Protein Complexes :

Dialyzed samples of nIF protein complexes were crosslinked through their cysteine residues by adding CuP_2 (Quinlan et al., 1986; Carden and Eagles, 1986; Kobashi, 1968) from a freshly prepared concentrated stock solution to a final concentration of 15 mM . Cross-linking was carried out for 20 min at room temperature and stopped by adding EDTA to 5 mM and iodoacetamide to 50 mM (modified from Quinlan et al., 1986; Carden and Eagles, 1986; Kobashi, 1968).

6– Analysis of Neuronal IF Protein Complexes by BN-PAGE:

Neuronal IF protein complexes were resolved by BN-PAGE as described (Schägger et al., 1994) with the following modifications: stacking gels were omitted; aminocaproic acid in the gels was replaced with 2 M urea; 5% glycerol was added to gel solutions used to pour gradient gels; the upper tank buffer contained 0.01% Coomassie Blue G-250; the 9-fold concentrated loading buffer contained 50 mM Bis Tris (pH 7.0), 2 M urea, 2.5% Coomassie Blue G-250 and 50% glycerol; gels were cast in a BioRad mini gel apparatus and

electrophoresed at 100 Volts. Dissociation of assembled protein complexes was achieved by heating the samples to 56°C for 4 min immediately prior to electrophoresis, which effectively prevented reassembly. Dissociation of disulfide-linked protein complexes was achieved by including 10 mM DTT in the heated samples. For second dimension gel electrophoresis, lanes were cut out of the gels after BN-PAGE and incubated in 1.5-fold concentrated SDS-sample buffer with or without 5% β-mercaptoethanol as described in the figure legends.

7– SDS Polyacrylamide Gel Electrophoresis :

Discontinuous gel electrophoresis of the samples on 4.5% or 6% SDS-polyacrylamide gels was performed as described (Laemli, 1970). Electrophoresis on 4% Weber and Osborn (1969) type gels was performed essentially as described (Sigma technical bulletin MWS-877X, 1988), except that regular Tris-containing SDS-sample buffer (see above) was used for dilution of samples.

8– Western Blot Analysis :

After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Montreal, Canada) as described (Athlan, 1996). After electroblotting of proteins resolved by BN-PAGE, PVDF membranes were rinsed in methanol to remove Coomassie Blue. Proteins were detected with specific, monoclonal antibodies (mAbs) against NFL (NR4), NFM (NN18), NFH (N52) (Sigma), peripherin (MAB1527) and α-internexin (MAB1525) (Chemicon International). Probing with mAbs, erasure of the blot for subsequent reprobing and visualization by enhanced chemiluminescence were conducted according to instructions provided by the manufacturer (NEN).

9– Coomassie Blue Staining and Quantification of Proteins :

Polyacrylamide gels were fixed for 10 to 20 min in destain solution (10% isopropanol, 10% acetic acid), stained overnight in Coomassie Blue solution

(0.1% Coomassie Blue R-250, 25% isopropanol, 10% acetic acid) (Fairbanks et al., 1971) and extensively destained against several changes of destain solution over a 24 hr period. Stained gels containing standardized amounts of rat NF proteins resolved alongside unknown samples were scanned and quantified with a BioImager apparatus (Millipore, Montreal, Canada). Subunit ratios were calculated using the following molecular masses for the rat NF subunits: NFL, 62 kD (Chin and Liem, 1989), NFM, 95 kDa (Napolitano et al., 1987), and NFH, 115 kDa (Chin and Liem, 1990).

RESULTS

1– nIF Proteins From Cultured DRG Neurons Resolved by BN-PAGE/2 M Urea Show Evidence of Heteromeric Associations :

We previously reported that α-internexin, peripherin and the NF triplet proteins in cultured DRG neurons were associated (Athlan et al., 1996) but the methods used could not accurately determine the precise oligomeric arrangement of these proteins. Further studies in our laboratory showed that nIF extracts resolved by BN-PAGE in the presence of increasing amounts of urea contained heterocomplexes of nIF proteins. In Fig.1, a DRG cytoskeletal pellet extracted with 2 M urea was electrophoresed using a "semi-native" version of BN-PAGE which included 2 M urea in the gel. Development of the resulting Western blots with mAbs against the individual nIF proteins showed seven major bands, some of which were immunoreactive with two of the mAbs. Based on the staining intensity and the relative mobilities of the various species, protein complexes 1 and 2 appeared to be heterooligomers of NFL with NFH or NFM, respectively, while complex 4 in the anti-NFL lane appeared to be an NFL dimer. Complex 3 in the anti-NFH lane was likely the hypophosphorylated NFH-containing equivalent of complex 1, since it was much more prominent in extracts from the cell body-enriched fraction obtained from localized DRG cultures (not shown), which we have shown to contain mostly this form of NFH (Athlan et al., 1996).The broad region designated as "6" contained species staining with mAbs against NFL, α-internexin and peripherin, suggesting the presence of homo- and/or heterodimers. Complex 4 in the anti-NFM lane, complex 5 in the anti NFL lane and complex 7 in the anti-peripherin lane could not be identified unambiguously. Initial cross-linking studies with further resolution in a second, SDS-PAGE dimension, appeared to confirm some of the associations described above and further studies were undertaken to fully characterize these and other small nIF protein complexes. This was achieved by mixing various combinations of individually purified nIF

subunits in the presence of 6 M gua-HCl, dialyzing against buffered 2 M urea, and resolving the resulting complexes on "semi-native" BN-PAGE gels prepared with the same buffer as the dialysis solutions.

2– Purified NFL forms heterodimers with NFH and NFM:

As shown in Fig.2 (lanes 1 and 3), purified NFH and NFM migrated on BN-PAGE/2 M urea as single bands with apparent molecular masses of 260 kDa and 214 kDa respectively. Heating to 56°C prior to electrophoresis did not affect their mobility (lanes 2 and 4) indicating that NFH and NFM migrated as monomers in this gel system, with anomalously high apparent molecular masses as is also observed on SDS-PAGE (Kaufmann et al., 1984). Gradual increases in the gel urea concentration to 8 M failed to produce smaller species, confirming the identity of the NFH and NFM bands as monomers (not shown).

NFL dialyzed and resolved on BN-PAGE/2 M urea (Fig.2, lane 5) yielded two bands with apparent molecular masses of 226 kDa and 94 kDa. Heating the dialyzed NFL to 56°C prior to electrophoresis (Fig.2, lane 6) converted almost all of the larger species to the more rapidly migrating form. Disulfide cross-linking of the NFL homooligomer through the unique cysteine residue in the α-helical domain (Chin and Liem, 1989) using CuP_2 (Quinlan et al., 1986) did not alter the migration pattern (lane 7) and effectively protected a significant portion of the NFL 226 kDa species from heat-induced conversion to the 94 kDa species (lane 8) indicating that these were homodimers and monomers respectively. The CuP_2-induced cross-links could be broken by including 10 mM DTT in the heated samples (lane 9) as would be expected for disulfide bonds.

When mixtures of NFL with NFH or NFM were analyzed by BN-PAGE/2 M urea, the bands previously identified in the lanes containing these proteins alone were all present. In addition, new complexes appeared with apparent molecular masses of 360 kDa for the NFH/NFL mixture (Fig.2, lane 10) and

328 kDa for the NFM/NFL mixture (lane 15), indicating the formation of NFH/NFL and NFM/NFL heterooligomers under these conditions. As was seen with the NFL homodimer, these heterooligomers were heat labile (lanes 11 and 16), they could be stabilized by CuP_2 cross-linking (lanes 13 and 18) and the disulfide cross-links were reduced with DTT (lanes 14 and 19).

Given the apparent anomalous migration of nIF proteins in this gel system, and because both NFH and NFM each contains more than one cysteine residue (Napolitano et al., 1987; Chin and Liem, 1990), two complementary approaches were used to confirm that the heterooligomers formed between NFL and NFH or NFM were heterodimers. In Fig.3, triplicate samples of NFH/NFL (panel A) and NFM/NFL (panel B) heterooligomers were prepared and resolved essentially as shown in Fig.2. The large amount of material used allowed direct visualization of all protein species in the first (BN-PAGE/2 M urea) dimension as they appeared as Coomassie Blue G-250-stained bands against a lighter blue background. The protein bands were excised, briefly equilibrated in SDS-sample buffer, resolved in the second dimension by discontinuous SDS-PAGE alongside standardized mixtures of NF subunits, visualized by Coomassie Blue R-250 staining and quantified with a BioImager apparatus (Millipore). As shown in Fig.3, the NFH/NFL and NFM/NFL heterooligomers purified by BN-PAGE in the first dimension broke down in the second SDS-containing dimension to give essentially equimolar amounts of NFH and NFL (ratio = 1.16 $\pm$ 0.02) or NFM and NFL (ratio = 1.06 $\pm$ 0.02) (mean $\pm$ standard deviation). These results clearly demonstrate that the observed heterooligomers are equimolar assemblies of their NF subunit constituents.

In Fig.4, NFH/NFL (panel A) and NFM/NFL (panel B) heterooligomers were formed, cross-linked and resolved by BN-PAGE/2 M urea essentially as shown in Fig.2. Some lanes were blotted onto a PVDF membrane and reacted with the anti-NF mAbs to reveal the positions of the various protein complexes in the first dimension as indicated at the top of the figure. Other lanes were cut

out of the gels, equilibrated in SDS-sample buffer without reducing agent and electrophoresed in the second dimension on a Weber and Osborne type gel (Weber and Osborne, 1969), which provided a better estimate of the molecular mass of cross-linked proteins than was obtained with Laemli type gels (data not shown), as previously reported (Quinlan et al.,1986; Carden and Eagles, 1986; Sigma technical bulletin MWS-877X, 1988; Bragg and Hou, 1975; Bretscher and Weber, 1980). Following transfer to a PVDF membrane, the blots were probed with an anti-NFL mAb (Fig.4, bottom part of panels A and B), erased, and reprobed with an anti-NFH (Fig.4, upper part of panel A) or an anti-NFM mAb (Fig.4, upper part of panel B). The cross-linked NFH/NFL hetero-complex seen in the first (BN-PAGE/2 M urea) dimension (H/L) resolved into three bands in the second, SDS-PAGE dimension. The spot labelled H/L in the second dimension reacted with both anti-NFH and anti-NFL mAbs (top and bottom part of panel A, respectively), confirming its heteromeric character. Non cross-linked NFH (H) and NFL (L) were also present in this second dimension, as could be expected from examining the heated, cross-linked samples in Fig.2 (lanes 13 and 18). The homodimeric NFL from the crosslinked sample seen in the first dimension (L/L) gave rise to some cross-linked (L/L) and monomeric (L) NFL in the second dimension while the NFH and NFL species that were monomeric in the first dimension (H, L) remained so in the second dimension (H, L). Similar results were obtained for the NFM/NFL mixture (Panel B) although cross-linked dimeric NFL (L/L) could not be adequately resolved from monomeric NFM (M), apparently due to upward streaking of the latter. It was also observed that the relative amount of cross-linked heterooligomer was lower on the Weber and Osborne type gels than would be predicted from the data in Fig.2 (e.g., lanes 13 and 18). This was a reproducible yet unexplained limitation of this gel system that was not observed when second dimension electrophoresis was carried out on a discontinuous SDS-PAGE system (not shown). The apparent molecular masses of the various species detected were determined and are listed in Table 1. The values for cross-linked NFH/NFL, NFM/NFL and NFL/NFL were within 3% of the summed molecular masses of

their monomeric constituents thereby confirming their dimeric character. Taken together, the results in Figs.2, 3 and 4 and in Table 1 conclusively demonstrate that under the conditions described here, NFH/NFL and NFM/NFL heterodimers and NFL/NFL homodimers are formed, and can be resolved by BN-PAGE/2 M urea.

3– α-Internexin and Peripherin Form Heterodimers With NF Subunits :

In Fig.5, α-internexin and peripherin were tested for their ability to dimerize alone or in combination with other nIF proteins. The experiments were carried out essentially as shown in Fig.2 except that cross-linking and heating of some species was omitted. Some of the previously characterized NF species are shown again to provide useful molecular mass landmarks. Based on the relative mobilities of the protein complexes, both α-internexin and peripherin formed homodimers (~159 kDa and ~143 kDa in lanes 5 and 7 of Fig.5, respectively) that broke down to the monomer species upon heat treatment (~73 kDa and ~66 kDa in lanes 6 and 8 respectively). The staining intensity of the heat treated peripherin sample was reproducibly lower than that of the unheated sample (lanes 8 and 7 respectively), apparently due to in-gel dimerization of the monomeric species leading to extensive streaking and concomitant loss of signal. The significant amount of dimeric peripherin in the heated sample and the virtual absence of monomeric peripherin in all of the other peripherin-containing lanes (lanes 7, 9 and 14-16) confirms its very strong tendency to dimerize under the conditions used. The various dimeric species obtained when mixing α-internexin and peripherin (lane 9) could not be resolved adequately on these gels and cross-linking experiments failed to show any heterodimeric interactions between these two proteins (not shown). Based on the relative mobility of the protein complexes, heterodimers containing α-internexin or peripherin with NFH (~356 kDa) or NFM (~320 kDa) (lanes 10, 11, 14 and 15 respectively) were also observed. Although the relative yields of these heterodimers were much lower than those observed for the

NFH/NFL and NFM/NFL species, markedly greater amounts could be generated by dialyzing and resolving the samples in 0.05 M urea instead of 2 M urea (not shown). It is not known why the α-internexin/NFH and α-internexin/NFM heterodimers in Fig.5 migrated as doublets although protein degradation seems unlikely since it was not observed in the purified subunit preparations (not shown). Mixing α-internexin or peripherin with NFL produced heterodimers that could not be resolved from the α-internexin and peripherin homodimers (lanes 12 and 16, respectively). However, erasure of the Western blot and reprobing with anti-NFL mAb alone clearly revealed the presence of α-internexin/NFL (~177 kDa, lane 13) and peripherin/NFL (~167 kDa, lane 17) heterodimers.

DISCUSSION

We recently reported that α-internexin, peripherin and the NF triplet proteins in cultured DRG neurons are associated (Athlan, 1996), thereby raising questions regarding the assembly stage during which such heteromeric associations can take place. Characterization of IF protein dimers has been hampered by a lack of practical and widely accessible techniques for resolving small oligomeric assembly intermediates. We have now shown that the recently reported BN-PAGE procedure (Schägger et al., 1994), modified to include urea, can provide direct and unambiguous separation of nIF protein complexes and allows multiple combinations of nIF proteins to be resolved in parallel. To this end, Fig.2 provides direct evidence for heterodimer formation between NFL and NFH or NFM, while Fig.5 further shows heterodimeric associations between α-internexin or peripherin and the individual NF subunits.

Small IF assembly intermediates have been generated by a variety of means, all revolving around the use of low ionic strength buffers, sometimes containing urea or guanidine in the 2-3 M range. Such buffers have been used either as a diluent of whole IFs (Carden and Eagles, 1986) or as the dialysis component for limited reassembly of IF proteins from concentrated guanidine or urea solutions (Quinlan et al., 1986; Hatzfeld and Weber, 1990; Steinert, 1990; Cohlberg et al., 1995; Cohlberg et al., 1987; Hisanaga et al., 1990). IF protein oligomers generated under these conditions have been considered to be valid assembly intermediates since addition of physiological salt concentrations coupled with removal of urea or guanidine if required, leads to the formation of normal appearing IFs. Moreover, reassembly of NFs (Zackroff et al., 1982; Liem and Hutchison, 1982), α-internexin (Chiu et al., 1989; Kaplan et al., 1990) and peripherin (Parysek and Goldman, 1987) from urea-containing buffers has been well documented. More specifically, NFL and NFM have been successfully co-assembled into native-like filaments by stepwise dialysis from

guanidine solution to a physiological buffer, including an intermediate step involving a buffered 2 M urea solution similar to ours (Cohlberg et al., 1995). Taken together, these data indicate that other nIF protein oligomers formed under such conditions would be valid assembly intermediates.

A previous report (Cohlberg et al., 1995) described the resolution of NF subunit heterotetramers containing equimolar amounts of NFL with NFH or NFM on "native" gels containing 2 M urea. In contrast, our study using the BN-PAGE system revealed that dimers were the predominant species under similar conditions. It is possible that the presence of Coomassie Blue G-250 destabilizes tetrameric species, thus favoring the dimers that we observed, although such effects were not reported by Schägger et al (1994). Alternatively, the bovine material used by Cohlberg et al. (1995) may form complexes that are more stable in 2 M urea than those formed by the rat nIF proteins utilized in our work. However, neither of these possibilities affects the present conclusions relating to heterodimer formation.

NFH and NFM did not interact with themselves (Fig. 2 and 5) or with each other (not shown), consistent with the many reports indicating that NFM and NFH do not assemble or co-assemble significantly *in vitro* (Zackroff et al., 1982; Liem and Hutchison, 1982; Geisler and Weber, 1981) or *in vivo* (Lee et al., 1993, Ching and Liem, 1993) and further indicate that the interactions we did observe were not likely to be due to non-specific interactions between related α-helical rod domains.

The first step in IF assembly involves the formation of an unstaggered, parallel, coiled-coil dimer (Steinert and Roop, 1988; Parry et al., 1985). Residues in positions "a" and "d" in the heptad repeats making up the α-helices are believed to form inward facing hydrophobic seams while the other residues face outward (reviewed in Cohen and Parry, 1990). NFL contains a unique cysteine residue located in a "d" position within a heptad repeat in the rod domain (Chin and Liem1989). Disulfide cross-linking of NFL homodimers and other IF protein dimers sharing this conserved residue has

been well documented and has provided an important argument favoring the unstaggered, parallel, coiled-coil model of IF homodimer structure (Quinlan et al., 1986; Cohen and Parry, 1990). However, disulfide bond formation between NFL and NFH or NFM under the conditions used here is somewhat puzzling. Indeed, the location of the closest cysteine residue in the rod domain of NFH in the linker region "L2", 70 residues removed from the cysteine residue in NFL, appears to preclude an unstaggered alignment of these proteins. A similar problem involves the cysteine in NFM occupying the "a" position in a heptad, 41 residues away from the cysteine in NFL. Yet, Fig.3 clearly demonstrated that cross-linked NFL/NFH and NFL/NFM dimers can be resolved as such in the BN-PAGE/2 M urea dimension raising the possibility that, in contrast to other IF proteins characterized so far (Fuchs and Weber, 1994; Steinert and Roop, 1988; Quinlan et al., 1986, Parry et al., 1985), NF subunits associated in a coiled-coil heterodimer may be in a staggered arrangement. This interpretation appears unlikely, considering that some twisting of the helices would also be required to adequately align the cysteine residues involved in the cross-linking of NFM or NFH to NFL. A more likely explanation may be that the 2 M urea-containing buffer used here allows for slippage and flexibility within the coiled-coil structure which would permit interactions between cysteine residues that are widely separated in the normal, unstaggered alignment. According to this view, the mobility of subunits in such loosely assembled coiled-coil dimers would still allow for more efficient intra-dimer cross-linking than for cross-linking between unassembled random collision complexes, which were never observed in these experiments.

α-Internexin and peripherin were shown in Figs. 5 to form heterodimers with NFH or NFM, suggesting that co-assembly of these proteins can take place at the dimer level. The comparatively greater propensity for NFL to form heterodimers with NFH and NFM further suggests that it is the preferred partner for these proteins. However, heterodimeric association of α-internexin or peripherin with the NF subunits may be important for cytoskeletal

reorganization during development and axonal regeneration. α-Internexin is the predominant nIF protein in developing central neurons (Kaplan et al., 1990; Fliegner et al., 1994) while peripherin expression is upregulated in regenerating, large caliber, NF-containing peripheral axons (Wong and Oblinger, 1990). In both instances, these α-internexin - or peripherin-containing networks are presumed to confer greater plasticity as reflected in the dynamic nature of the growing axon (Nixon and Shea, 1992). The ability of NF proteins to dimerize with α-internexin and peripherin could play an important role in allowing a gradual transition to take place from a more plastic α-internexin or peripherin-containing network to a stable, NF-enriched IF network in the mature axon (Giasson et al., 1997).

The modified version of BN-PAGE described here is a widely accessible, economical and versatile method that allows several different samples to be analyzed in parallel. Future work in our laboratory will focus on monitoring early nIF protein assembly intermediates and on assessing the effects of mutations or post-translational modifications known or suspected to affect IF assembly.

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REFERENCES

Athlan, E. S., Sacher, M. S. and Mushynski, W.E. (1997), Associations between intermediate filament proteins expressed in cultured dorsal root ganglion neurons. *J. Neurosci. Res.*, **47**, 300-310.

Bragg, P.D. and Hou, C. (1975) Subunit composition, function, and spatial arrangement in the Ca^{++} and Mg^{++} activated adenosine triphosphatases of *Escherichia coli* and *Salmonella typhimurium*. *Arch. Biochem. Biophys.* **167**, 311-321.

Bretscher, A. and Weber, K. (1980) Villin is a major protein of the microvillus cytoskeleton which binds both G and F actin in a calcium dependent manner. *Cell* **20**, 839-847.

Carden, M.J. and Eagles, P.A.M. (1986) Chemical cross-linking analyses of ox neurofilaments. *Biochem. J.* **234**, 587-591.

Carpenter, D.A. and Ip, W. (1996) Neurofilament triplet protein interactions: evidence for the preferred formation of NFL containing dimers and a putative function for the end domains. *J. Cell Science,* **109**, 2493-2498.

Chadan, S., Moya, K.L., Portier, M.-M. and Filliatreau, G. (1994) Identification of a peripherin dimer: changes during axonal development and regeneration of the rat sciatic nerve. *J. Neurochem.* **62**, 1894-1905.

Chin, S.S.M. and Liem, R.K.H. (1989) Expression of rat neurofilament proteins NF-L and NF-M in transfected non-neuronal cells. *Eur. J. Biochem.* **50**, 475-490.

Chin, S.S.M. and Liem, R.K.H. (1990) Transfected rat high-molecular-weight neurofilament (NF-H) coassembles with vimentin in a predominantly nonphosphorylated form. *J. Neurosci.* **10**, 3714-3726.

Ching, G.Y. and Liem, R.K.H. (1993) Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. *J. Cell Biol.* **122**, 1323-1335.

Chiu, F.C., Barnes, E.A., Das, K., Haley, J., Socolow, P., Macaluso, F.P. and Fant, J. (1989) Carachterization of a novel 66 Kd subunit of mammalian neurofilaments. *Neuron* **2**, 1435-1445.

Cohen, C. and Parry, D.A.D. (1990) α-Helical coiled coils and bundles: how to design an ∝-helical protein. Proteins: *Structure, Function and Genetics* **7**, 1-15.

Cohlberg, J.A., Hajarian, H. and Sainte-Marie, S. (1987) Discrete soluble forms of middle and high molecular weight neurofilament proteins in dilute aqueous buffers. *J. Biol. Chem.* **262**, 17009-17015.

Cohlberg, J.A., Hajarian, H., Tran, T., Alipourjeddi, P. and Noveen, A. (1995) Neurofilament protein heterotetramers as assembly intermediates. *J.Biol.Chem.* **270**, 9334-9339.

Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Electrophoretic analysis of the major polypeptide of the human erythrocyte membrane. *Biochemistry* **10**, 2606-2617.

Fliegner, K.H., Kaplan, M.P., Wood, T.L., Pintar, J.E. and Liem, R.K.H. (1994) Expression of the gene for the neuronal intermediate filament protein α-internexin coincides with the onset of neuronal differentiation in the developing rat nervous system. *J.Comp.Neurol.* **342**, 161-173.

Fuchs, E. and Weber, K. (1994) Intermediate filaments: structure, dynamics, function and disease. *Annu. Rev. Biochem.* **63**, 345-382.

Geisler, N. and Weber, K. (1981) Self-assembly *in vitro* of the 68,000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate sized filaments. *J. Mol. Biol.* **151**, 565-571.

Giasson, B.I. and Mushynski, W.E. (1997) Developmentally regulated stabilization of neuronal intermediate filament in rat cerebral cortex. *Neurosci. Lett.* **229**, 1-4.

Giasson, B.I. and Mushynski, W.E. (1996) Aberrant stress-induced phosphorylation of perikaryal neurofilaments, *J. Biol. Chem.* **271** 30404-30409.

Hatzfeld, M. and Weber, K. (1990) The coiled-coil of in vitro assembled keratin filaments is a heterodimer of type I and II keratins: use of site specific mutagenesis and recombinant protein expression. *J. Cell Biol.* **110**, 1199-1210.

Hisanaga, S., Ikai, A. And Hirokawa, N., J. (1990) Molecular architecture of the neurofilament. *J. Mol. Biol.* **211**, 857-869.

Hoffman, P.N. and Lasek, R.J. (1975) The slow component of axonal transport; Identification of major structural polypeptides of the axona and their generality among mammalian neurons. *J. Cell Biol.* **66**, 351-356.

Julien, J.-P. and Mushynski, W.E. (1982) Multiple phosphorylation sites in mammalian neurofilament polypeptides. *J. Biol. Chem.* **257**, 10467-10470.

Kaplan, M.P., Chin, S.S.M., Fliegner, K.H. and Liem, R.K.H. (1990) α-Internexin, a novel neuronal intermediate filament protein, precedes the low molecular weight neurofilament protein (NF-L) in the developing rat brain. *J. Neurosci.* **10**, 2735-2748.

Kaufmann, E., Geisler, N. And Weber, K. (1984) SDS-PAGE strongly overestimates the molecular masses of the neurofilament proteins. *FEBS Lett.* **170**, 81-84.

Kobashi, K. (1968) Catalytic oxidation of sulfhydryl groups by 0-phenanthroline copper complex. *Biochem. Biophys. Acta* **158**, 239-245.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, M. K., Xu, Z., Wong, P. C. and Cleveland, D. W., Neurofilaments are obligate heteropolymers in vivo, *J. Cell. Biol.*, **122** (1993) 1337-1350.

Leung, C.L. and Liem, R.K.H. (1996) Characterization of interactions between the neurofilament triplet proteins by the yeast two-hybrid system. *J. Biol. Chem.* **271**, 14041-14044.

Liem R.K.H. and Hutchison, S.B. (1982) Purification of individual components of the neurofilament triplet: filament assemly from the 70 000-Dalton subunit. *Biochemistry* **21**, 3221-3226.

Mulligan, L., Balin, B.J., Lee, V.M.-Y. and Ip, W. (1991) Antibody labelling of bovine neurofilaments: implications on the structure of neurofilament sidearms. *J. Struct. Biol.* **106**, 145-160.

Napolitano, E.W., Chin, S.S.M., Colman, D.R. and Liem, R.K.H. (1987) Complete amino acid sequence and *in vivo* expression of rat NF-M, the middle molecular weight neurofilament protein. *J. Neurosci.* **7**, 2590-2599.

Nixon, R. A. and Shea, T.B. (1992) Dynamics of neuronal intermediate filaments: a developmental perspective, *Cell Motil. Cytoskeleton* **22**, 81-91.

Parry, D.A.D., Steven, A.C. and Steinert, P.M. (1985) The coiled-coil molecules of intermediate filaments consist of two parallel chains in exact axial register. *Biochem. Biophys. Res. Commun.* **127**, 1012-1018.

Parysek, L.M. and Goldman, R.D. (1987) Characterization of intermediate fialments in PC12 cells. *J. Neurosci.* **7**, 781-791.

Parysek, L.M., McReynolds, M.A., Goldman, R.D. and Ley, C.A. (1991) Some neural intermediate filaments contain both peripherin and the neurofilament proteins. *J.Neurosci.Res.* **30**, 80-91.

Portier, M.-M., de Néchaud, B. and Gros, F. (1984) Peripherin, a new member of the intermediate filament protein family. *Dev.Neurosci.* **6**, 335-344.

Quinlan, R.A., Hatzfeld, M. and Franke, W.W. (1986) Characterization of dimer subunits of intermediate filament proteins. *J.Biol.Chem.* **192** 337-349.

Schägger, H., Cramer, W.A., and von Jagow, G. (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Analyt. Biochem.* **217**, 220-230.

Sokoloff, R.L. and Frigon, R.P. (1981) Rapid spectrophotometric assay of dodecyl sulfate using acridine orange. *Anal. Biochem.* **118**, 138-141.

Steinert, P.M. and Roop, D.R. (1988) Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.* **57**, 593-625.

Steinert,P.M. (1990) The two-chain coiled-coil molecule of native keratin intermediate filaments is a type I-type II heterodimer. *J. Biol. Chem.* **265**, 8766-8774.

Sun, D., Macioce, P., Chin, S.S.M. and Liem, R.K.H. (1997) Assembly properties of amino- and carboxyl-terminally truncated neurofilament NF-H proteins with NF-L and NF-M in the presence and absence of vimentin. *J. Neurochem.* **68**, 917-926.

Weber, K. and Osborn, M.J. (1969)The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406-4412.

Wong, J. and Oblinger, M.M., J. (1990) Differential regulation of peripherin and neurofilament gene expression in regenerating rat DRG neurons. *Neurosci. Res.* **27**, 332-341.

Zackroff, R.V., Idler, W.W., Steinert, P.M. and Goldman, R.D. (1982) In vitro reconstitution of intermediate filaments from mammalian neurofilament triplet polypeptides. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 754-757.

Protein Species	Relative Molecular Mass (kDa)	Predicted Molecular Mass (kDa)
H/L complex	308	317
M/L complex	239	246
L/L complex	158	156
H (monomeric)	239	
M (monomeric)	168	
L (monomeric)	78	

Table I : Relative molecular masses of the various species resolved by two-dimensional electrophoresis after cross-linking of NFH/NFL and NFM/NFL mixtures. The samples were cross-linked and resolved by BN-PAGE followed by SDS-PAGE as described in Experimental Procedures and in the legend to Fig.4. H, M and L denote the NF subunits NFH, NFM and NFL respectively.

Figure 1: Western blot analysis of DRG cytoskeletal extracts. Cytoskeletal pellets were purified from the axonal compartment of localized rat DRG cultures, extracted with buffered 2 M urea, resolved by BN-PAGE/2 M urea on a 4%-8% polyacrylamide gradient gel and electroblotted onto a PVDF membrane. Strips were then cut out and reacted with mAbs against the individual nIF proteins as indicated at the top of the figure. H, M, L, I, P are strips reacted with mAbs against NFH, NFM, NFL, α-internexin and peripherin, respectively. The major protein complexes detected are numbered 1 to 7 and are discussed further in the text.

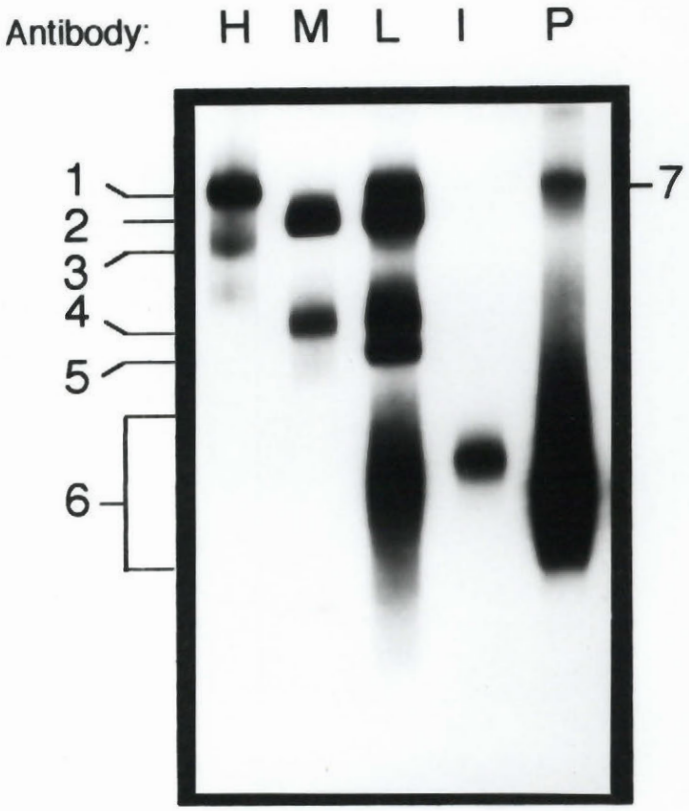

Antibody:
H
M
L
I
P
1
2
3
4
5
6
7

Figure 2: Western blot analysis of associations between purified NF subunits following dialysis against buffered 2 M urea. NF subunits were individually purified, mixed as indicated at the top of each panel, dialyzed against buffered 2 M urea, and left untreated or were treated with CuP_2, heating to 56°C and/or 10 mM DTT as indicated at the bottom of each lane. Samples were then resolved by BN-PAGE in 2 M urea on a 4%-8% polyacrylamide gradient gel. The Western blot was developed with a mixture of mAbs against the individual NF subunits. H, M, and L denote monomeric NF subunits NFH, NFM and NFL, respectively. H/L, M/L, and L/L are dimeric species containing the indicated NF subunits.

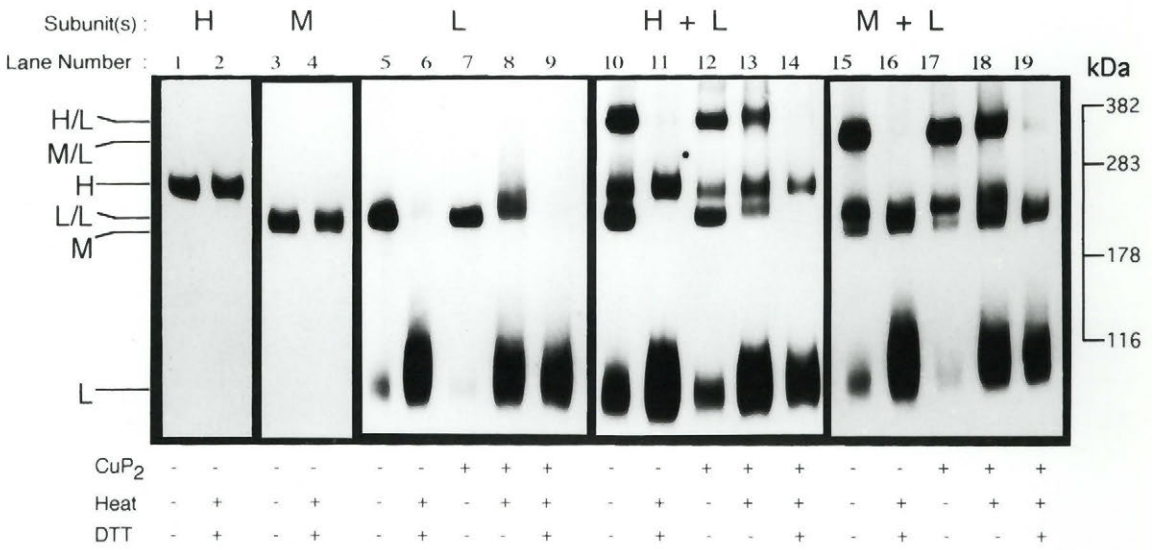

Subunit(s) : H M L H + L M + L
Lane Number : 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
kDa
382
283
178
116
H/L
M/L
H
L/L
M
L
CuP2 - - - - - - + + + - - + + + - - + + +
Heat - + - + - + - + + - + - + + - + - + +
DTT - + - + - + - - + - + - - + - + - - +

Figure 3: Determination of NF subunit stoichiometries in heterooligomers by two-dimensional gel electrophoretic analysis. Individually purifed NFH and NFL (panel A) or NFM and NFL (panel B) were mixed, dialyzed against buffered 2 M urea and resolved by BN-PAGE/ 2 M urea on a 4%-8% polyacrylamide gradient gel. The putative heterodimeric species were located and the protein bands were cut out, briefly equilibrated in sample buffer, layered on a 6% polyacrylamide gel, resolved into monomeric constituents by discontinuous SDS-PAGE (23) and stained with Coomassie Blue R-250 as described in Experimental Procedures. H, M, and L denote the monomeric NF subunits NFH, NFM and NFL respectively.

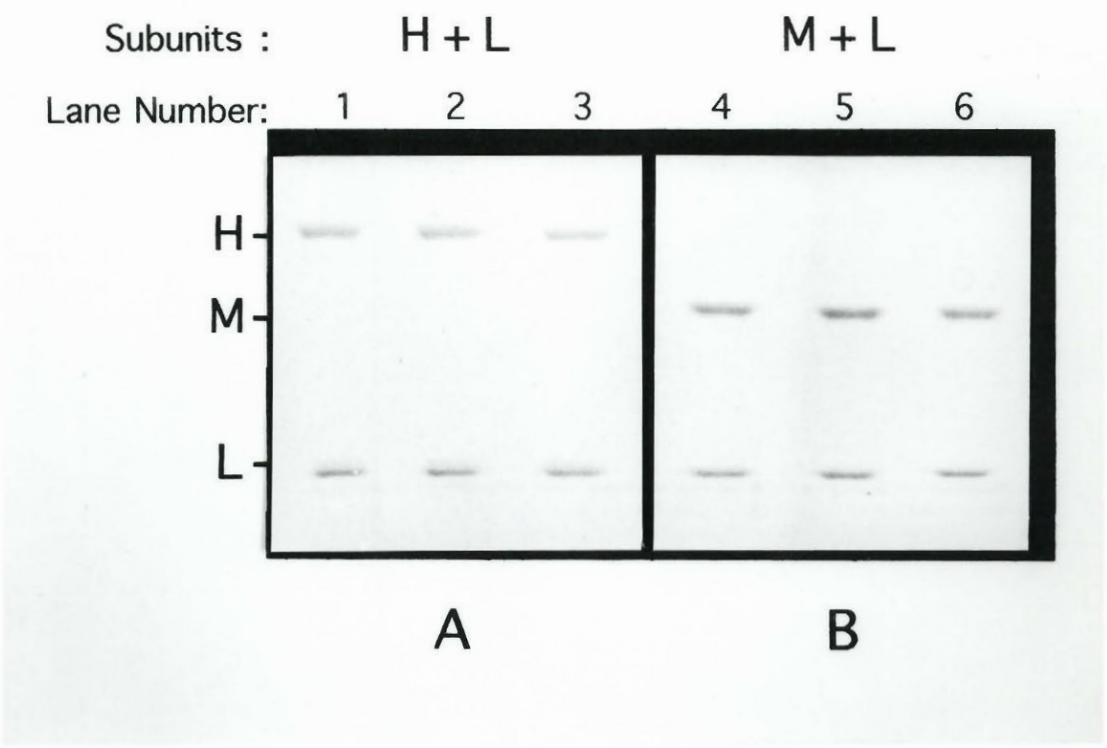

Subunits :
H + L
M + L
Lane Number:
1
2
3
4
5
6
H
M
L
A
B

Figure 4: Two-dimensional gel electrophoretic determination of the relative molecular masses of crosslinked NF heterooligomers. Individually purifed NFH and NFL (panel A) or NFM and NFL (panel B) were mixed, dialyzed against buffered 2 M urea, cross-linked with CuP_2 as described in Experimental Procedures and resolved by BN-PAGE/2 M urea on a 4%-8% polyacrylamide gradient gel. Replicate lanes were immunoblotted to identify the species indicated at the top of each figure. Other lanes were briefly equilibrated in SDS-sample buffer (without reducing agent), layered on a 4% polyacrylamide Weber and Osborne (27) type gel and resolved alongside purified NF subunits and molecular weight markers covering the range 66-487 Kd. The blots were probed with an anti-NFL Ab (bottom panels in A and B), erased and reprobed with anti-NFH and anti-NFM mAbs (top panels in A and B respectively). The letters identifying the various species are defined in the legend to Fig.2.

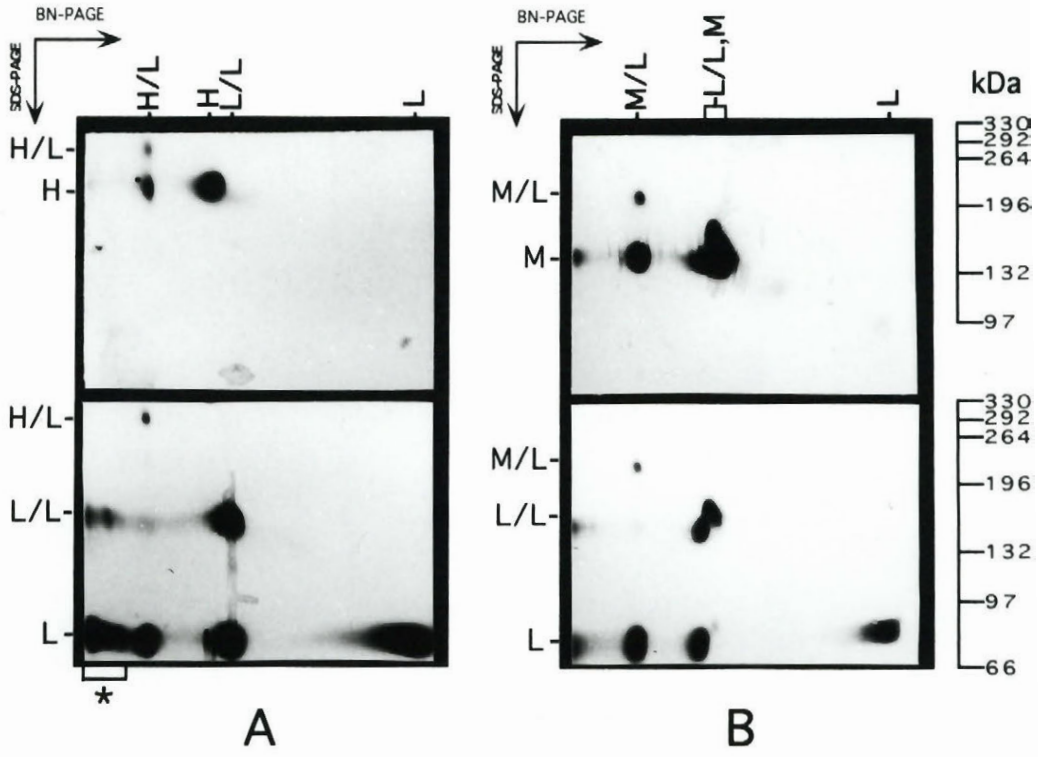
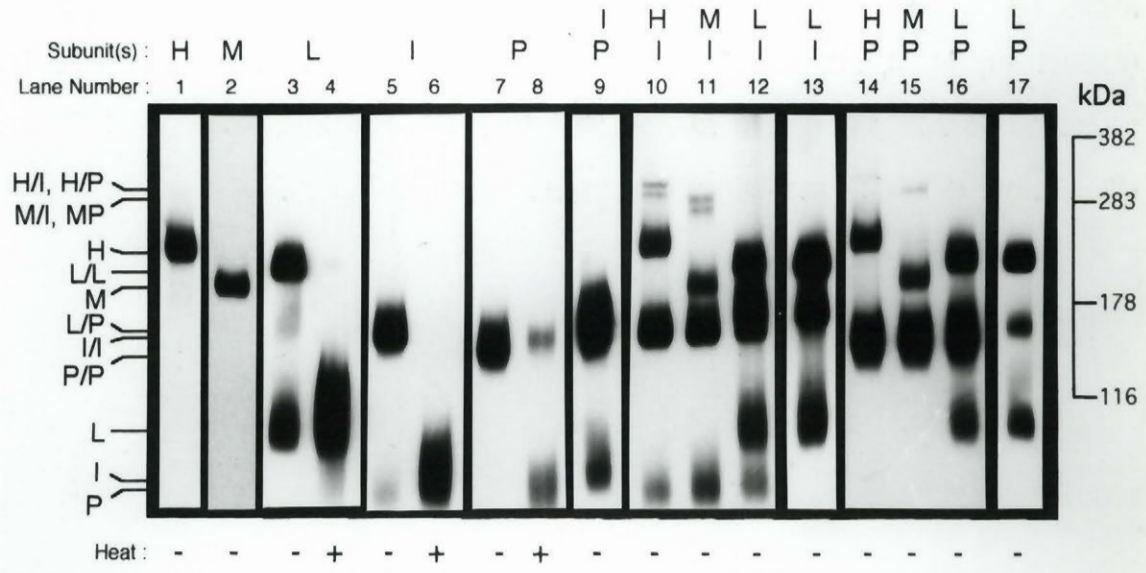

BN-PAGE
SDS-PAGE
H/L
H
L/L
L
H/L
H
H/L
L/L
L
*
A
BN-PAGE
SDS-PAGE
M/L
L/L,M
L
kDa
330
292
264
196
132
97
M/L
M
330
292
264
196
132
97
66
M/L
L/L
L
B

Figure 5: Western blot analysis of associations between purified nIF subunits following dialysis against buffered 2 M urea. nIF subunits were individually purified, mixed as indicated at the top of each panel, dialyzed against buffered 2 M urea and some samples were heated to 56°C as indicated at the bottom of each lane. Samples were then resolved by BN-PAGE/2 M urea on a 4%-8% polyacrylamide gradient gel. The Western blot was developed with a mixture of mAbs against the individual nIF subunits. Lanes 12 and 16 were erased, reprobed with the anti-NFL mAb only, and are shown in lanes 13 and 17, respectively. H, M, L, I, and P denote nIF monomeric subunits NFH, NFM, NFL, α-internexin and peripherin, respectively. L/L, H/I, M/I, L/I, I/I, H/P, M/P, L/P and P/P denote dimeric species containing the various nIF proteins.


Subunit(s) : H M L I P I P H I M I L I L I H P M P L P L P
Lane Number : 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
kDa
382
283
178
116
H/I, H/P
M/I, MP
H
L/L
M
L/P
I/I
P/P
L
I
P
Heat : - - - + - + - + - - - - - - - - -

CHAPTER 4

NEUROFILAMENT PROTEIN DIMER STABILITY

Eric S. Athlan and Walter E. Mushynski

ABSTRACT

The present study stems from our previous report indicating that the light neurofilament (NF) subunit (NFL) forms both homodimers as well as heterodimers with the heavy (NFH) and midsized (NFM) NF subunits during the initial stage of NF assembly (Athlan and Mushynski, 1997). We now report that the relative stability of NF protein dimers varies according to modifications present on NFL as determined by native polyacrylamide gel electrophoresis in a lateral urea concentration gradient. Posttranslational modifications present on spinal cord NFL and presumably lacking in the bacterially expressed protein, destabilized its homodimeric interactions in favor of heterodimeric interactions with NFH and NFM, perhaps to ensure proper dimer stoichiometry for NF assembly. A mutation in NFL suspected of interfiering with NF assembly in motor neurons (Lee et al., 1994) was also shown to destabilize NFL containing dimers indicating that this mutation impacts on the earliest stage of NF assembly. The lateral urea gradient/BN-PAGE sytem described here constitutes a useful tool for assessing the effects of various forms of modification on intermediate filament protein interactions at the biochemical level.

INTRODUCTION

Most mammalian cells contain 8-10 nm-wide filaments composed of subunits belonging to the large family of intermediate filament (IF) proteins. These IF proteins share a common domain organization consisting of an amino-terminal head domain and a carboxy-terminal tail domain flanking a highly conserved α-helical rod domain (Fuchs and Weber, 1994). Post mitotic neurons contain IFs known as neurofilaments (NFs), which are made up of three subunits, NFL (light), NFM (medium) and NFH (heavy) (Hoffman and Lasek, 1975). They may also express one or both of two other IF proteins, α-internexin (Chiu et al., 1989; Kaplan et al., 1990) and peripherin (Portier et al., 1984), the latter being found in neurons with axons that project to the periphery (Escurat et al., 1990). These five neuronal IF proteins are co-expressed in cultured, embryonic dorsal root ganglion (DRG) neurons, where they form a highly integrated network (Athlan et al., 1997).

The advent of DNA techniques for manipulating the sequences of genes and cDNAs encoding NF proteins has led to significant advances in our knowledge of factors governing filament assembly (Heins and Aebi, 1994). NFs are now known to be obligate heteropolymers *in vivo* (Lee et al., 1993; Ching and Liem, 1993; Nagakawa et al., 1995) and deletion mutagenesis has helped to define the roles of the head, rod and tail domains of the individual subunits in filament formation (Gill et al., 1990; Wong and Cleveland, 1990; Ching and Liem, 1993; Heins et al., 1993; Lee et al., 1993; Nakagawa et al., 1995; Sun et al., 1997). However, the effects of these various subunit alterations have generally been assessed by procedures such as immunofluorescence and electron microscopy. These approaches can detect gross perturbations in IF formation but provide little information about the actual stage of the assembly process being affected by a given deletion or point mutation.

We have recently reported that co-assembly of the five neuronal IF proteins present in cultured DRG neurons can occur as early as the coiled-coil

dimerization stage (Athlan and Mushynski, 1997). The procedure used in that study was a version of "blue" native polyacrylamide gel electrophoresis (BN-PAGE) (Schägger et al., 1994) modified to contain urea. We have further adapted this economical and versatile method to analyze the stability of NF protein complexes in a continuum of steadily increasing denaturing conditions. Thus, by carrying out BN-PAGE in gels containing a lateral urea concentration gradient, we were able to compare the stabilities of NFL/NFL, NFL/NFM and NFL/NFH coiled-coil dimers as well as to gauge the effects of a Leu→Pro mutation in the rod domain of NFL (Lee et al., 1994) on NFL homo- and heterodimer stability. The results clearly indicate that the strength of NFL dimer associations can be modulated and that BN-PAGE can be used to assess the effects of IF subunits alterations at the biochemical level.

MATERIALS AND METHODS

1– Materials :

Acrylamide:bis acrylamide 37.5:1 and low melting type agarose were purchased from BioShop (Hamilton, ON). Ammonium persulfate, BisTris, and specific monoclonal antibodies (mAbs) against NFH (N52), NFM (NN18) and NFL (NR4) were from Sigma (St.Louis, MO). Specific mAbs against peripherin (MAB1527) and α-internexin (MAB1525) were from Chemicon International (Temecula, CA). Glycerol and Whatman filter paper were from Fisher Scientific. Urea and SDS were from ICN Biomedical (Mississauga, ON).

2– Purification Of nIF Subunits From Rat Spinal Cord :

NFH, NFM, NFL, α-internexin and peripherin subunits were purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Athlan and Mushynski, 1997).

3– Bacterial Expression and Mutagenesis of NFL :

Using the Muta-Gene M13 *in vitro* mutagenesis kit (BioRad, CA), an adenine to cytosine conversion was effected at the +4 position of a murine NFL cDNA clone(Julien et al., 1985) in order to place the start codon within a unique NcoI restriction site. This cDNA was propagated into the pBluescript (KS^+) vector (Stratagene, La Jolla, CA), linearized by cleavage at a unique SacII site downstream of the NFL termination codon, repaired, and ligated to HindIII linkers. Following cleavage with NcoI and HindIII, the NFL cDNA was gel-purified, ligated into the corresponding restriction sites of the pET23d vector (Novagen Inc, Madison, WI), and the pET23d/NFL DNA was transfected into competent *Escherichia coli* HS174 (DE3) cells. NFL expression was induced by addition of 1 mM isopropyl-β-Thiogalactoside (IPTG) followed by a 3 hour

incubation of the bacterial cultures at 37°C. The cells were then pelleted, resuspended in 0.8 volume of 25% sucrose, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0, 2 mM phenylmethylsulfonylfluoride (PMSF), and lysed on ice for 30 min after adding lysozyme to 0.17 mg/ml. The lysate was then adjusted to 10 mM $MgCl_2$, 1mM $MnCl_2$, 1 mg/ml DNAseI, incubated a further 30 minutes on ice, and mixed with 1 vol of 0.2 M NaCl, 1% deoxycholate, 1% Nonidet P40, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, 2 mM PMSF. Following a 30 minute centrifugation at 5,000xg, the pelleted inclusion bodies were resuspended in 0.5% Triton X-100, 1 mM EDTA, 2 mM PMSF and recentrifuged at 5,000xg for 30 minutes. This step was repeated 2 more times and the inclusion bodies were then solubilized in adsorbtion buffer (8 M urea, 10 mM $NaPO_4$ pH 7.4, 2 mM PMSF) using a dounce homogenizer. The suspension was centrifuged for 30 minutes at 10,000xg and the bacterially expressed NFL (NFL^b) in the supernatant was adsorbed, batchwise, to hydroxylapatite pre-equilibrated in adsorbtion buffer. The hydroxylapatite was then washed twice with 5 volumes of adsorbtion buffer and three times with 5 volumes of 8 M urea, 70 mM $NaPO_4$, pH 7.0, 2 mM PMSF. NFL^b was eluted in 2 volumes of 8 M urea, 300 mM $NaPO_4$, pH 7.0, and its purity (>95%) was confirmed by SDS-PAGE.

NFL containing the L394P mutation (NFL^{L394P}) (Lee et al., 1994) was prepared using a two-stage PCR approach. Outer primers spanned the Kpn I (5' primer) and Sac I (3' primer) restriction sites in NFL cDNA, which are approximately 160 bp apart. The overlapping mutagenic primers spanning codon 394 were : 5'-AAAACTCCCGGAAGGCGAAGAGACC-3' (5' primer) and 5'-TCGAATGTCTTTTGAGGGCCTTCCG-3' (3' primer). The altered bases are underlined.

Stage 1 PCR amplification using pET23d/NFL as the template involved two samples, one containing the 5' outer primer and the 3' mutagenic primer, and the other consisting of the 5' mutagenic primer and the 3' outer primer. Following 30 cycles of amplification with Taq polymerase, the ~80 bp products were gel purified, extracted, combined and supplied with the 5' and 3' outer primers only for stage 2 PCR. The ~160 bp stage 2 product was cleaved with

Sac I and Kpn I, gel purified, and ligated into the corresponding sites of pET23d/NFL from which the non-mutated 160 bp fragment had been removed. Sequencing confirmed the presence of the expected mutation in pET23d/NFL^{L394P} which was used for bacterial expression and purification of NFL^{L394P} as for NFL^{b} (see above).

4– Partial Assembly of nIF Proteins :

Individually purified nIF proteins were partially reassembled by dialysis against buffered 50 mM or 1 M urea as described previously (Athlan and Mushynski, 1997).

5– Analysis of nIF Protein Complexes by BN-PAGE :

BN-PAGE of large nIF protein complexes reassembled in buffered 50 mM urea was carried out essentially as described (Athlan and Mushynski, 1997), except that the urea and acrylamide concentrations were lowered to 50 mM and 3.2 % respectively. These gels were strengthened by adding 1% low melting type agarose to the gel mixture.

6– Analysis of nIF Protein Complexes By BN-PAGE/Lateral Urea Gradient Gels :

BN-PAGE gels containing a linear 1-4 M urea gradient in a lateral configuration were poured in a BioRad minigel apparatus using a mixture of "solution 1" and "solution 2". Solution 1 contained 50 mM BisTris (pH 7.0), 1 M urea, 4% acrylamide:bisacrylamide (37.5:1) and 1% saturated bromophenol blue in water to monitor the uniformity of the gradient. Solution 2 contained 50mM BisTris (pH 7.0), 4 M urea, 5% glycerol and 4% acrylamide:bisacrylamide (37.5:1). Both solutions were prepared, aliquoted, and kept at -20 °C. They were thawed at room temperature and chilled to 0 °C before use. Polymerization was initiated by adding 0.05% ammonium

persulfate and 0.15% TEMED. The bottom of the mini gel assembly which consisted of glass plates, 1.5 mm spacers, and clamps, was sealed with adhesive tape and a 1 ml plug of 8% polyacrylamide, 50 mM BisTris (pH 7.0) was poured and allowed to polymerize. The excess liquid was blotted with Whatman 3 MM filter paper. A spacer was then inserted at the top of the assembly and melted 1% agarose in water was used to seal the joints. The assembly was then placed on a side edge and gel solutions 1 and 2 contained in two compartments of a gradient generator were mixed to form a linear gradient that was introduced through a 2 mm opening left at the top of the inserted spacer. When the liquid reached the top, the assembly was briefly tilted to avoid trapping air bubbles against the lateral (but currently "top") spacer. After polymerization, the assembly was placed back on its bottom edge and the top spacer was removed. A stacking gel was then poured using solution 1 only, and a 15 well comb was inserted.

7– Western Blot Analysis :

nIF proteins resolved by BN-PAGE/50 mM urea and BN-PAGE with a lateral 1-4 M urea gradient were transferred to a polyvinylidene fluoride (PVDF) membrane and detected with specific mAbs by enhanced chemiluminescence as described previously (Athlan et al., 1997, Athlan and Mushynski, 1997). The detected proteins were quantified using a BioImager apparatus (Millipore).

RESULTS

We previously reported that except for NFH and NFM mixtures, all pairwise combinations of the individually purified nIF proteins NFH, NFM, NFL, α-internexin and peripherin, resulted in the formation of heterodimeric species upon dialyzing against buffered 2 M urea (Athlan and Mushynski, 1997). Given that NFs (Liem and Hutchison, 1982), α-internexin (Chiu et al., 1989; Kaplan et al., 1990; Balin and Miller, 1995), peripherin (Parysek and Goldman, 1987) and other IF proteins (Hedrick and Smith, 1968; Steinert et al., 1976) can form filaments when dialyzed from urea-containing solutions, it was inferred that larger protein complexes would be generated by lowering the urea concentration in the dialysis solution. Consequently, various combinations of nIF proteins were dialyzed and analyzed by BN-PAGE/urea and Western blotting as previously reported (Athlan and Mushynski, 1997) except that the urea concentration in the dialysis solution and in the gel was reduced to 50 mM. Effective resolution of the resulting large protein complexes was ensured by the use of 3.2% acrylamide gels strengthened with agarose. Size determination of the nIF protein complexes was not attempted due to a lack of suitable molecular mass markers. As shown in Fig.1, NFM and possibly NFH formed very small amounts of putative homodimeric species. Moreover, the NFM monomer migrated more slowly than the NFL dimer in these more porous gels, a reversal of the situation observed on 6% polyacrylamide gels (Athlan and Mushynski, 1997). The latter phenomenon has been reported previously for other proteins and PAGE systems (Cohlberg et al., 1987; Cohlberg et al., 1995). NFL and peripherin alone formed mainly their respective homodimers, traces of larger oligomers and a prominent streak at the top of the gel, suggesting the rapid formation of very large oligomers. Mixtures of NFH or NFM with NFL, α-internexin or peripherin showed putative heterotetrameric species in addition to the previously identified heterodimers (Athlan and Mushynski, 1997). Mixing NFL with α-internexin increased the streaking at the top of the gel,

relative to the lanes with these proteins alone, again suggesting the rapid formation of very large oligomers. Mixtures of NFL with peripherin did not reveal any additional complexes. Interestingly, α-internexin had a unique behavior in that it displayed a well-defined ladder pattern starting at the dimer level and including 5-8 different species.

Progressively decreasing the urea concentration from 2 M urea, where only dimers can be detected, to 50 mM urea failed to produce larger amounts of approximately tetramer-size assembly intermediates (data not shown). Given that nIF protein dimers can readily be caracterized by BN-PAGE, it was of interest to determine whether dimerization constitutes a regulatory step in the nIF protein assembly process. Should this be the case, nIF protein dimer stability may be affected by post-translational modifications and mutations known or suspected to regulate assembly.

The relative stability of NF protein dimers consisting of NF subunits from various sources was thus tested on BN-PAGE gels containing a lateral urea gradient. Individually purified NFH, NFM and NFL from spinal cord (NFL^{s}) contained post-translational modifications representative of *in vivo* assembled NFs. Bacterially expressed NFL (NFL^{b}) was assumed to lack these post-translational modifications and had indeed been shown not to contain phosphate moieties (M.G. Sacher, unpublished observation) in contrast to the situation for spinal cord NFL, which contains 2-3 moles of phosphate per mole of polypeptide (Julien and Mushynski, 1982). A bacterially expressed NFL bearing a Leu→Pro mutation (NFL^{L394P}) known to affect NF assembly *in vivo* (Lee et al., 1994) was also used to assess the impact of this mutation on dimer formation.

The three different types of NFL were dialyzed against 1 M urea, individually or in combination with NFH or NFM, and analyzed on BN-PAGE gels containing a lateral 1-4 M urea gradient. The protein complexes were electroblotted onto a PVDF membrane and detected by Western blot analysis with an anti-NFL mAb. As shown on Figs.2-5, the protein complexes gradually

dissociated as the urea concentration increased. The relative dissociation of the various NF protein complexes was quantified and plotted against the deduced urea concentration in the lanes after setting the first sample (at the lowest urea concentration) as 100%. As shown on Fig.2, NFL^{b} homodimers remained relatively stable up to a urea concentration of ~2.8 M, after which they gradually dissociated down to 50% of the initial level (D_{50}) at ~3.4 M urea and ~35% of the initial level at 3.8 M urea. NFL^{s} showed a sigmoidal dissociation curve, with a plateau extending form 1.9 M to 2.7 M urea and reaching D_{50} at ~2.8 M urea, and <10% of its initial level at 3.8 M urea. NFL^{L394P} showed a profile similar to NFL^{s}, except that D_{50} was reached at ~1.8 M instead of ~2.8 M urea. Using both D_{50} and the extent of dissociation in 3.8 M urea as measures of homodimer stability, NFL^{s} was clearly less stable than NFL^{b} under these conditions, suggesting that post-translational modifications on NFL^{s} reduce homodimeric interactions. Using similar criteria, NFL^{L394P} homodimers were clearly less stable than both NFL^{s} and NFL^{b} homodimers.

The dissociation profile of heterodimers containing NFM with the different forms of NFL is shown on Fig.3. NFL^{b}/NFM heterodimers showed a gradual and steady dissociation profile reaching D_{50} at ~2.4 M urea and <10% association at ~3.2 M urea. NFL^{s}/NFM heterodimers showed similar values but the dissociation profile had a more sigmoidal shape, with a slight plateau between 1.9 M and 2.4 M urea. NFL^{L394P}/NFM heterodimers had a lower D_{50} of ~1.9 M urea and were almost fully dissociated by ~2.7 M urea, indicating that the mutation also destabilized this heterodimeric association.

The dissociation profiles of heterodimers containing NFH with the different forms of NFL, shown on Fig.4, resemble the NFL/NFM profiles in Fig.3 although there are some differences. For instance, the D_{50} values for NFL^{b}/NFH, NFL^{s}/NFH and NFL^{L394P}/NFH were approximately 2.1M, 1.8 M and 1.7 M urea, respectively. Moreover, the plateau for NFL^{s}/NFH extended from 1.6 M to 2.3 M urea and the dissociation profile that followed showed a very gradual decline to <10% at ~3.0 M urea. This contrasts with NFL^{b}/NFH, and

NFL^{L394P}/NFH heterodimers which were at less than 10% of the maximum level by 2.5 M and 2.1 M urea, respectively, suggesting that a small fraction of NFL^{S} contains post-translational modifications that stabilize heteromeric associations with NFH. NFL^{L394P}/NFH heterodimers were clearly less stable than the other NFL/NFH heterodimers, indicating that the mutation also has a destabilizing effect on NFL/NFH interactions.

In Fig.5, the data from Figs.2-4 were plotted so as to compare the dissociation profiles for the individual forms of NFL homo- and heterodimers with NFH and NFM. As shown in Fig.5b, the NFL^{b} homodimer is far more stable than the NFL^{b} /NFM and NFL^{b} /NFH heterodimers, with a roughly 1.0 M to 1.4 M urea concentration difference at D_{50}. This is in sharp contrast to NFL^{S} in Fig.5a where the maximum difference in urea concentration at D_{50} between the various dimers species was 0.8 M urea. This indicates that post-translational modification of NFL^{S} confers a more comparable stability between the various NFL-containing assembly intermediates. As shown in Fig.5c, all three types of NFL^{L394P}-containing dimers showed a reduced stability with a urea concentration of ~1.7 to 2.0 M urea at D_{50}.

DISCUSSION

Following our previous report on homo- and heterodimerization of nIF protein subunits upon dialysis against buffered 2 M urea (Athlan and Mushynski, 1997), we report here that following dialysis against lower urea concentrations, most combinations of nIF proteins form both dimers and very large oligomers, with little accumulation of intermediate species. We have hypothesized that dimer formation may be modulated by events such as posttranslational modification or mutation of the subunits suspected of affecting nIF assembly. This hypothesis was tested by using three different versions of the NFL protein in a lateral urea gradient-BN-PAGE assay. NFL^{s} possessed the postranslational modifications associated with this protein in adult rat spinal cord while NFL^{b} was assumed to be unmodified, and NFL^{L394P} contained a mutation suspected to affect its assembly properties (Fuchs and Coulombe, 1992; Lee et al., 1994). The data indicate that the dissociation profile of dimer species involving various versions of the NFL protein with or without the other NF subunits varies significantly.

All dimer species formed with NFL^{L394P} were of lower stability than the ones formed with either NFL^{s} or NFL^{b} suggesting that this mutation has an effect on the very first stage of NF assembly. NF protein dimerization involves amino acid heptad repeats of the form $(abcdefg)_n$ where apolar residues at positions "a" and "d" form a hydrophobic seam that mediates coiled-coil interactions (reviewed in Cohen and Parry, 1990). A minimum of 4 heptad repeats was shown to confer a stable dimeric association between artificial peptides (Lau et al., 1984; Su et al., 1994). Given that the rod domain of NF proteins contains about 35 heptad repeats (Geisler et al., 1984), it is noteworthy that a point mutation located so as to disrupt only the last heptad at the end of the coil region should have such a drastic impact on dimer formation. Perhaps coiled-coil dimer formation involves a cooperative effect that allows a single disruption to affect the entire assembly. Nevertheless, the data clearly confirms biochemically the assembly-disrupting effect of mutations in

the highly conserved region at the end of coil 2, as was observed *in vivo* for NFL (Lee et al., 1994) and for the cytokeratins (Fuchs and Coulombe, 1992). The data also indicated that dimer formation did in fact occur with NFL^{L394P} and that dimerization was only affected kinetically. In fact, Lee et al. (1994) noted that NFs appeared normal in transgenic animals expressing the L394P mutation, although there was a complete reorganization of the neuronal cytoskeleton. Thus, subsequent stages of assembly do occur and may also be affected by the L394P mutation. Our data do not preclude such a possibilty although the precise mechanism at work is still speculative. For instance, the partially disrupted coiled-coil NFL^{L394P} dimer may not be as readily packed into filaments as the wild type protein, resulting in macroscopically normal-appearing NFs with altered properties. Regardless of the mechanism involved, it is understood that the end result of a modification that affects assembly would be the sum total of the various effects impacting upon the individual stages of assembly.

A number of post-translational modifications have been reported for the various NF proteins, including phosphorylation (reviewed in Fuchs and Weber, 1994) and *O*-linked glycosylation (Dong et al., 1993; 1996). Although NF head domain phosphorylation appears to regulate assembly *in vitro* (Gonda et al., 1990; Hisanaga et al., 1994) and *in vivo* (Sihag and Nixon, 1991; Sacher et al., 1994; Giasson et al., 1996) the precise mechanism by which it accomplishes this remains to be elucidated. Interestingly, NFL^{b} homodimers were much more stable than the ones formed by NFL^{s} whereas the heterodimers formed by both proteins with NFH and NFM were of comparable stability. These results imply that some posttranslational modification present on NFL^{s} but absent on NFL^{b} alters its association kinetics and increases the likelihood of heterodimer formation between NFL^{s} and NFH or NFM relative to the formation of NFL^{s} homodimers. This, in turn, would allow proper assembly of NFs from the appropriate proportions of the different dimeric species. If this hypothesis is correct, aberrant posttranslational modifications could have effects similar to

those obtained by altering NF subunit stoichiometries, which has been shown to result in NF accumulation in some transgenic animals that overexpress one of the NF subunits (Côté et al., 1993; Xu et al., 1993; Wong et al., 1995). Indeed, a direct link between posttranslational modifications of NF proteins and aberrant assembly is important in light of the fact that NF accumulations can have a causative effect in motor neuron diseases (Côté et al., 1993; Xu et al., 1993). The aberrant phosphorylation o fperikaryal NFH by stress-activated kinases may lead to such aggregate formation, although direct evidence for this is still lacking (Giasson and Mushynski, 1996; 1997)

The method presented here provides a powerful approach for studying at the biochemical level, IF protein modifications that modulate assembly by affecting intersubunit interactions.

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REFERENCES

Athlan, E.S. and Mushynski, W.E. (1997) Heterodimeric associations between neuronal intermediate filament proteins. *J. Biol. Chem.* **272**, 31073-31078.

Athlan, E.S., Sacher, M.S. and Mushynski, W.E. (1997) Associations between intermediate filament proteins expressed in cultured dorsal root ganglion neurons. *J. Neurosci. Res.*, **47**, 300-310.

Balin, B.J. and Miller, M.E. (1995) Reassembly of the 66 KDa neurofilament protein *in vitro* following isolation and purification from bovine spinal cord. *J.Neurosci.Res.* **40**, 79-88

Ching, G.Y. and Liem, R.K.H. (1993) Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. *J. Cell Biol.* **122**, 1323-1335.

Chiu, F.C., Barnes, E.A., Das, K., Haley, J., Socolow, P., Macaluso, F.P. and Fant, J. (1989) Carachterization of a novel 66 Kd subunit of mammalian neurofilaments. *Neuron* **2**, 1435-1445.

Cohen, C. and Parry, D.A.D. (1990) α-helical coiled coils and bundles: how to design an α-helical protein. *Prot. Struct. Func. Genet.* **7**, 1-15.

Cohlberg, J.A., Hajarian, H. and Sainte-Marie, S. (1987) Discrete soluble forms of middle and high molecular weight neurofilament proteins in dilute aqueous buffers. *J. Biol. Chem.* **262**, 17009-17015.

Cohlberg, J.A., Hajarian, H., Tran, T., Alipourjeddi, P. and Noveen, A. (1995) Neurofilament protein heterotetramers as assembly intermediates. *J.Biol.Chem.* **270**, 9334-9339.

Côté, F., Collard, J.-F. and Julien, J.-P. (1993) Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. *Cell* **73**, 35-46.

Dong, D.L.-Y., Xu, Z.-S, Chevrier, M.R., Cotter, R.J., Cleveland, D.W. and Hart, G.W. (1993) Glycosylation of mammalian neurofilaments: localization of

multiple *O*-linked *N*-acetylglucosamine moieties on neurofilament polypeptides L and M. *J. Biol. Chem.* **268**, 16679-16687.

Dong, D.L.-Y., Xu, Z.-S, Hart, G.W. and Cleveland, D.W. (1996) Cytoplasmic *O*-GlcNAc modification of the head domain and the KSP repeat motif of the neurofilament protein neurofilament-H. *J. Biol. Chem.* **271**, 20845-20852.

Escurat, M., Djabali, K., Gumpel, M., Gros, F. and Portier, M.-M. (1990) Differential expression of two neuronal intermediate filament proteins, peripherin and the low molecular mass neurofilament protein (NF-L), during the development of the rat. *J.Neurosci.* **10**, 764-784.

Fuchs, E. and Coulombe, p.A. (1992) Of mice and men: genetic skin diseases of keratin. *Cell* **69**, 899-902.

Fuchs, E. and Weber, K. (1994) Intermediate filaments: structure, dynamics, function, and disease. *Annu. Rev. Biochem.* **63**, 345-382.

Geisler, N., Fisher, S., Vandekerckhove, J., Plessmann, U. and Weber, K. (1984) Hybrid character of a large neurofilament protein (NFM): intermediate filament type sequences followed by a long acidic carboxy-terminal extension. *EMBO J.* **3**, 2701-2706.

Giasson, B.I. and Mushynski, W.E. (1996) Aberrant stress-induced phosphorylation of perikaryal neurofilaments. *J. Biol. Chem.* **271**, 30404-30409.

Giasson, B.I. and Mushynski, W.E. (1997) Study of proline-directed protein kinases involved in phosphorylation of the heavy neurofilament subunit. *J. Neurosci.* **17**, 9466-9472.

Giasson, B.I., Cromlish, J.A., Athlan, E.S., and Mushynski, W.E. (1996) Activation of cyclic AMP-dependent protein kinase in okadaic acid-treated neurons potentiates neurofilament fragmentation and stimulates phosphorylation of Ser-2 in the low-molecular-mass neurofilament subunit. *J. Neurochem.* **66**, 1207-1213.

Gill, S.R., Wong, P.C., Monteiro, M.J. and Cleveland, D.W. (1990) Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NFL) subunit. *J. Cell Biol.* **111**, 2004-2019.

Gonda, Y., Nishizawa, K., Ando, S., Kitamura, S., Minoura, Y., Nishi. Y. and Inagaki, M. (1990) Involvement of protein kinase C in the regulation of assembly-diassembly of neurofilaments *in vitro*. *Biochem. Biophys. Res. Commun.* **167**, 1316-1325.

Hedrick, J.L. and Smith, A.J. (1968) Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Arch. Biochem. Biophys.* **126**, 155-164.

Heins, S. and Aebi, U. (1994) Making heads and tail of intermediate filament assembly, dynamics and networks. *Curr. Opin. Cell Biol.* **6**, 25-33.

Heins, S., Wong, P.C., Müller, S., Goldie, K. and Cleveland, D.W. (1993) The rod domain of NFL determines neurofilament architecture, whereas the end domains specify filament assembly and network formation. *J. Cell. Biol.* **123**, 1517-1533.

Hisanaga, S., Matsuoka, Y., Nishizawa, K., Saito, T., Inagaki, M. and Hirokawa, N. (1994) Phosphorylation of native and reassembled neurofilaments composed of NFL, NFM, and NFH by the catalytic subunit of cAMP-dependent protein kinase. *Mol. Biol. Cell* **5**, 165-172.

Hoffman, P.N. and Lasek, R.J. (1975) The slow component of axonal transport: identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* **66**, 351-366.

Kaplan, M.P., Chin, S.S., Fliegner, K.H. and Liem, R.K.H. (1990) α-Internexin, a novel neuronal intermediate filament protein, precedes the low molecular weight neurofilament protein (NFL) in developing rat brain. *J. Neurosci.* **10**, 2735-2748.

Lau, S.Y.M., Taneja, A.K. and Hodges, R.S. (1984) Effect of chain length on the stabilization and formation of two-stranded α-helical coiled coils. *J. Biol. Chem.* **259**, 13253-13261.

Lee, M.K., Marszalek, J.R. and Cleveland, D.W. (1994) A mutant neurofilament subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. *Neuron* **13**, 975-988.

Lee, M.K., Xu, Z, Wong, P.C. and Cleveland, D.W. (1993) Neurofilaments are obligate heteropolymers *in vivo*. *J. Cell Biol.* **122**, 1337-1350.

Liem R.K.H. and Hutchison, S.B. (1982) Purification of individual components of the neurofilament triplet: filament assemly from the 70 000-Dalton subunit. *Biochemistry* **21**, 3221-3226.

Nakagawa, T., Chen, J., Zhang, Z., Kanai, Y. and Hirokawa, N. (1995) Two distinct function of the carboxyl terminal tail domain of NFM upon neurofilament assembly: cross-bridges formation and longitudinal elongation of filaments. *J. Cell Biol.* **129**, 411-429.

Parysek, L.M. and Goldman, R.D. (1987) Characterization of intermediate fialments in PC12 cells. *J. Neurosci.* **7**, 781-791.

Portier, M.-M., de Néchaud, B. and Gros, F. (1984) Peripherin, a new member of the intermediate filament protein family. *Dev.Neurosci.* **6**, 335-344.

Renner, W., Franke, W.W., Schmid, E., Geisler, N., Weber, K. and Mandelkow, E. (1981) Reconstitution of inetermediate-sized filaments from denatured monomeric vimentin. *J. Mol. Biol.* **149**, 285-306.

Sacher, M.G., Athlan, E.S. and Mushynski, W.E. (1994) Increased phosphorylation of the amino-terminal domain of the low molecular weight neurofilament subunit in okadaic acid-treated neurons. *J. Biol. Chem.* **269**, 14480-14484.

Schägger, H., Cramer, W.A., and von Jagow, G. (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Analyt. Biochem.* **217**, 220-230.

Sihag, R.K. and Nixon R.A. (1990) *In vivo* phosphorylation and dephosphorylation of the 145 KDa subunit of neurofilaments: evidence for regulation by second messenger-dependent protein kinases. *J. Biol. Chem.* **265**, 4166-4171.

Su, J.Y., Hodges, R.S. and Kay, C.M. (1994) Effect of chain length on the formation and stability of synthetic α-helical coiled coils. *Biochemistry* **33**, 15501-15510.

Sun, D., Macioce, P., Chin, S.S.M. and Liem, R.K.H. (1997) Assembly properties of amino- and carboxyl-terminally truncated neurofilament NFH proteins with NFL and NFM in the presence and absence of vimentin. *J. Neurochem.* **68**, 917-926.

Xu, Z., Cork, L.C., Griffin, J.W. and Cleveland, D.W. (1993) Increased expression of neurofilament subunit NFL produces morphological alterations that resemble the pathology of human motor disease. *Cell* **73**, 23-33.

Wong, P.C. and Cleveland, D.W. (1990) Characterization of dominant and recessive assembly-defective mutations in mouse neurofilament NFM. *J. Cell Biol.* **111**, 1987-2003.

Figure 1: Western blot analysis of associations between purified nIF subunits following dialysis against 50 mM urea. nIF subunits were individually purified, mixed as indicated at the top of each lane, dialyzed against buffered 50 mM urea and resolved by BN-PAGE/50 mM urea on a 3.2% polyacrylamide gel strengthened with 1% agarose. The Western blot was probed with a mixture of mAbs against the individual nIF subunits except for lanes 4 and 5 which were probed with mAbs against α-internexin and peripherin, respectively. H, M, L, I, and P denote the monomeric nIF subunits NFH, NFM, NFL, α-internexin and peripherin, respectively. L/L, H/L, M/L, H/I, M/I, L/I, I/I, H/P, M/P, L/P and P/P denote dimeric species containing the various nIF proteins. H/H and M/M denote putative homodimeric species containing these proteins.

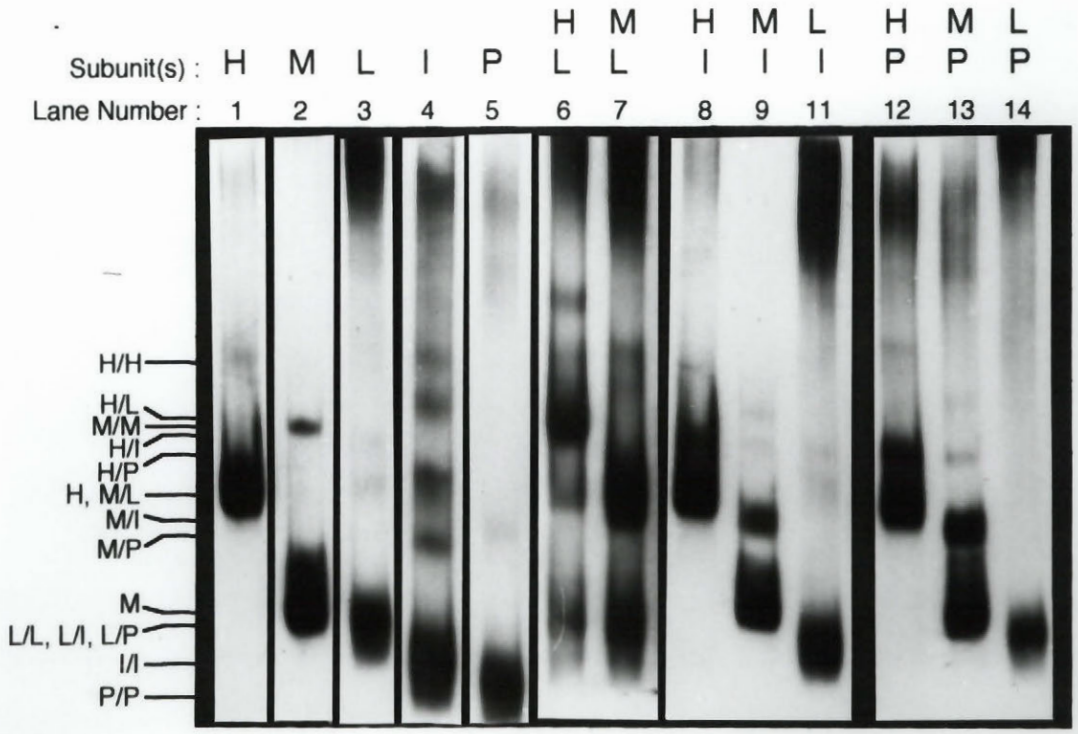
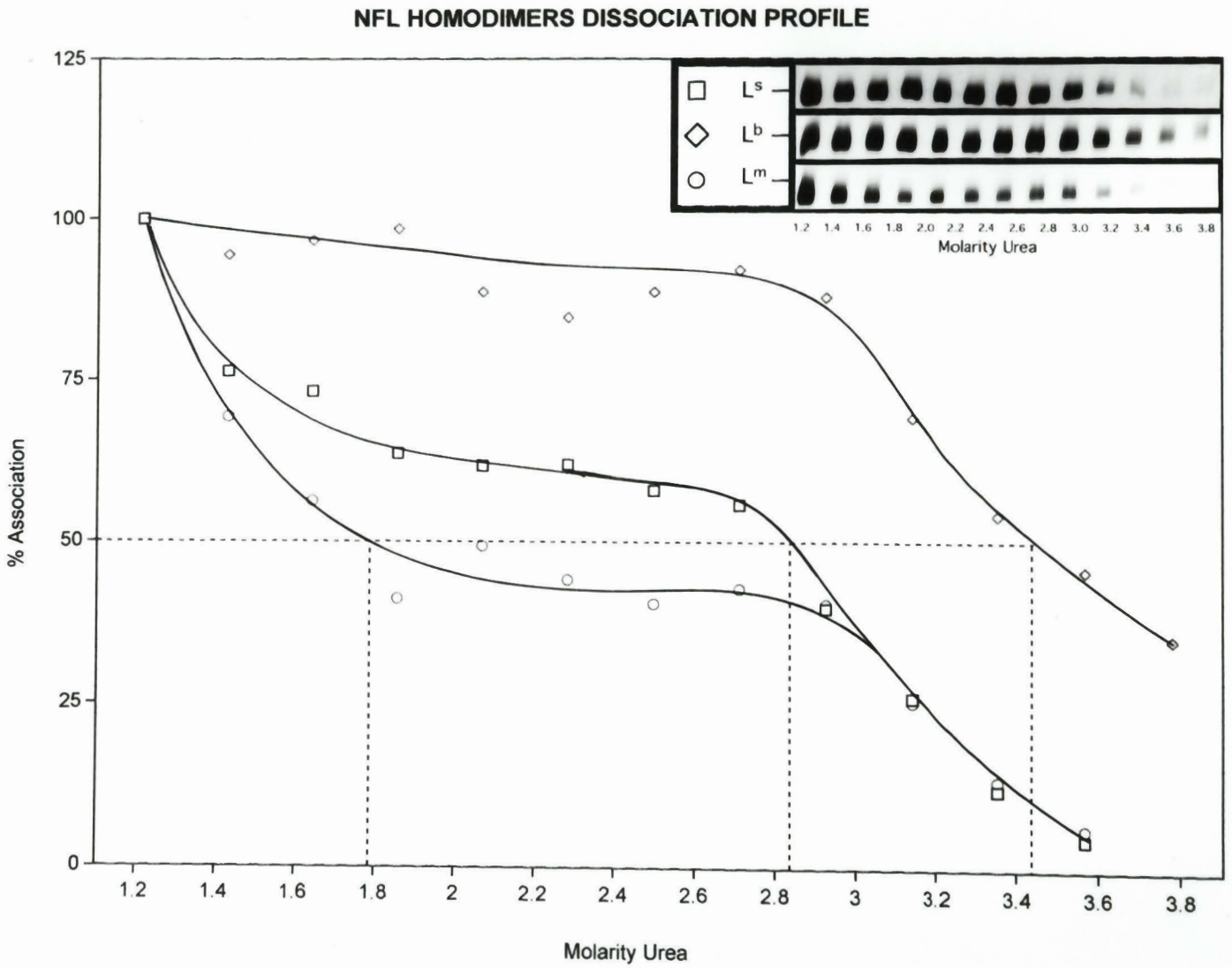

Subunit(s) : H M L I P H L M L H I M I L I H P M P L P
Lane Number : 1 2 3 4 5 6 7 8 9 11 12 13 14
H/H
H/L
M/M
H/I
H/P
H, M/L
M/I
M/P
M
L/L, L/I, L/P
I/I
P/P

Figure 2: Western blot analysis of the dissociation profile of NFL homodimers. NFL was dialyzed against buffered 1 M urea and resolved by BN-PAGE on 4% polyacrylamide gels containing a lateral 1-4 M urea concentration gradient. The Western blot was developed with an anti-NFL mAb and the amount of undissociated NFL dimers was quantified, arbitrarily setting the amount at the lowest urea concentration as 100%. A best fit curve was drawn and the urea concentration corresponding to 50% dissociation was estimated. L^s, refers to spinal cord NFL while L^b and L^m refer to bacterially expressed wild type and L394P mutant NFL, respectivley.


NFL HOMODIMERS DISSOCIATION PROFILE
125
100
75
50
25
0
% Association
1.2
1.4
1.6
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2
2.2
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Ls
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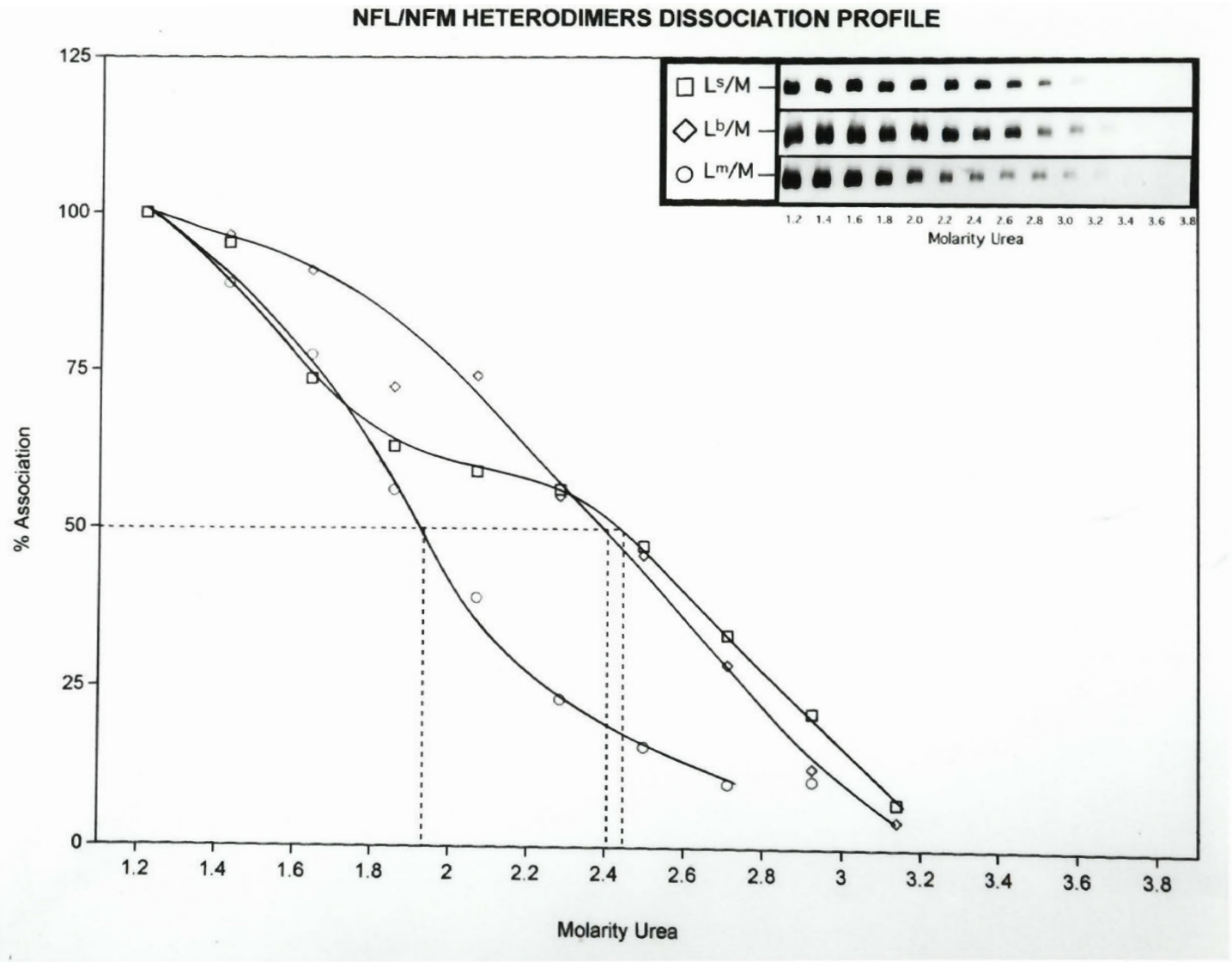
Lm
1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8
Molarity Urea

Figure 3: Western blot analysis of the dissociation profile of NFL/NFM heterodimers. NFL and NFM were mixed, dialyzed against buffered 1 M urea and resolved by BN-PAGE on 4% polyacrylamide gels containing a lateral 1-4 M urea concentration gradient. The Western blot was developed with an anti-NFL mAb and the amount of undissociated NFL/NFM heterodimers was quantified, arbitrarily setting the amount at the lowest urea concentration as 100%. A best fit curve was drawn and the urea concentration corresponding to 50% dissociation was estimated. L^s/M, L^b/M and L^m/M refer to NFM-containing heterodimers with the various species of NFL described in the legend to Fig.2.


NFL/NFM HETERODIMERS DISSOCIATION PROFILE
% Association
125
100
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50
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0
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1.4
1.6
1.8
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2.2
2.4
2.6
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3
3.2
3.4
3.6
3.8
Molarity Urea
Ls/M
Lb/M
Lm/M
Molarity Urea

Figure 4: Western blot analysis of the dissociation profile of NFL/NFH heterodimers. NFL and NFH were mixed, dialyzed against buffered 1 M urea and resolved by BN-PAGE on 4% polyacrylamide gels containing a lateral 1-4 M urea concentration gradient. The Western blot was developed with an anti-NFL mAb and the amount of undissociated NFL/NFH heterodimers was quantified, arbitrarily setting the amount at the lowest urea concentration as 100%. A best fit curve was drawn and the urea concentration corresponding to 50% dissociation was estimated. L^s/H, L^b/H and L^m/H refer to NFH-containing heterodimers with the various species of NFL described in the legend to Fig.2.

NFL/NFH HETERODIMERS DISSOCIATION PROFILE

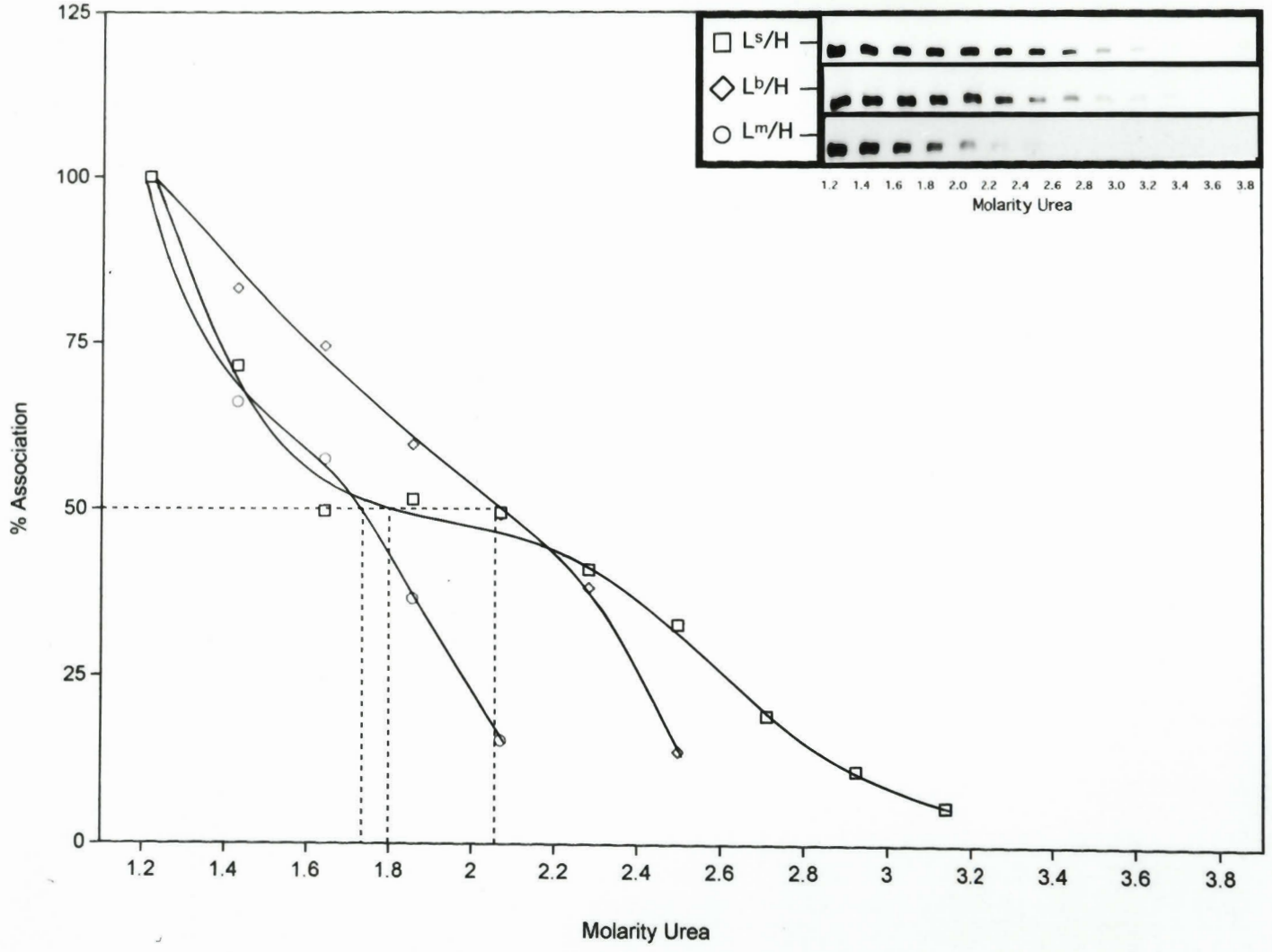
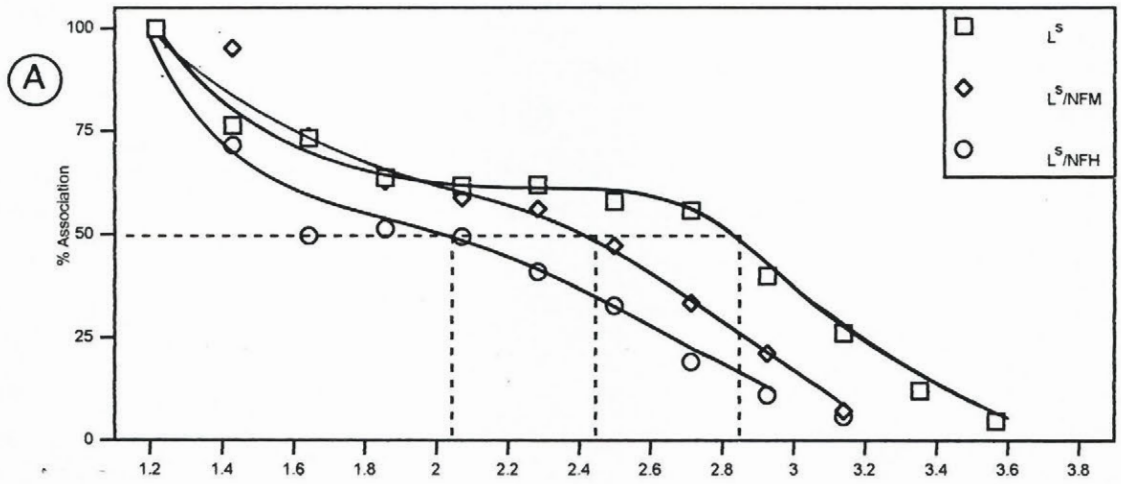
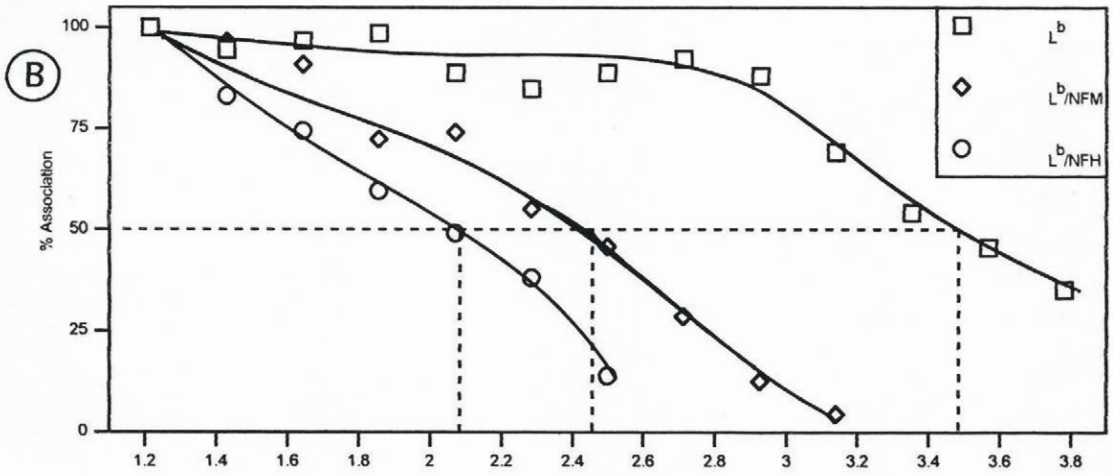


Figure 5: The data presented in Figs.2-4 were plotted on three separate graphs comparing the stability of individual NFL-containing species. A) Spinal cord NFL-containing NF dimers. B) Wild type, bacterially expressed, NFL-containing species. C) bacterially expressed, L394P mutant, NFL-containg species. The various species are labelled as in the legend to Fig.2-4.

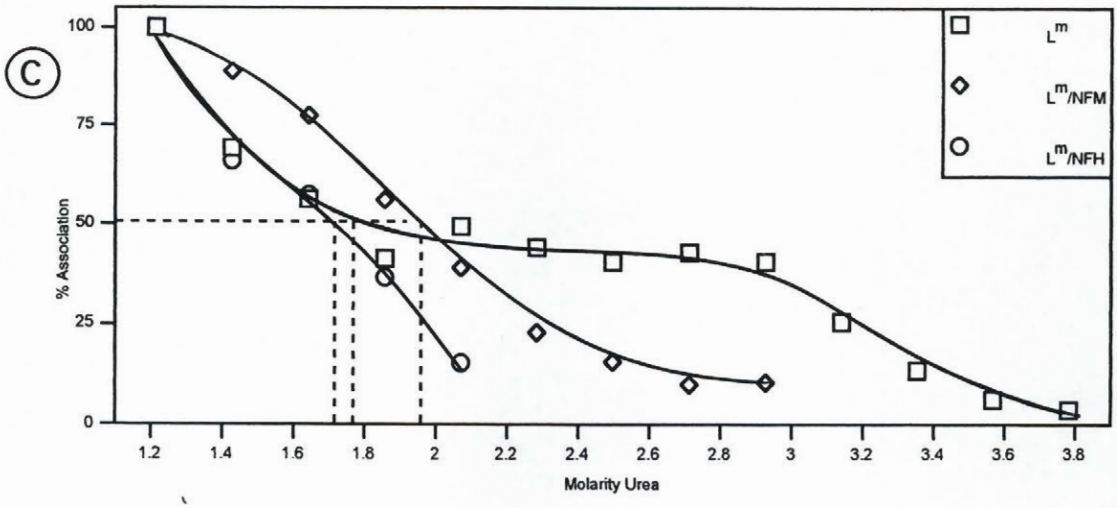
SPINAL CORD NFL DIMERS DISSOCIATION PROFILES



BACTERIALLY EXPRESSED NFL DIMERS DISSOCIATION PROFILES



MUTANT NFL DIMERS DISSOCIATION PROFILE



GENERAL DISCUSSION

When the studies detailed in this thesis were initiated, various approaches reviewed in chapter 1 had already yielded a wealth of information regarding nIF protein assembly. However, several questions remained that were beyond the scope and capabilities of the available technology. The work reported here has helped resolve some of these questions and has provided fresh tools uniquely suited to the study of nIF proteins assembly.

The report by Sacher et al., (1992) that the shellfish toxin okadaic acid triggers NF disassembly in cultured neurons provided a novel approach to study NF dynamics. In addition, the fact that mAbs specific for the individual NF subunits could co-immunoprecipitate other NF subunits in extracts from okadaic acid-treated neurons suggested a way to probe the nature of disassembly intermediates as a means to further understand heteropolymeric assembly of nIF subunits.

The data presented in Chapter 2 indicated that all five nIF proteins, NFH, NFM, NFL, α-internexin and peripherin, were co-localized in cultured DRG neurons. Several aspects of the developmental pattern of expression of these proteins in cultured neurons were consistent with results of *in vivo* studies. These included expression of α-internexin early in development (Fliegner et al., 1994), co-expression of NFL and NFM, (Carden et al., 1987) and delayed expression of NFH (reviewed in Lindenbaum et al., 1988). However there were also some differences. Unlike the *in vivo* situation (Escurat et.al., 1990), peripherin levels were not tightly coordinated with the levels of NFL. Moreover, cultured DRG neurons retained a high level of α-internexin expression, without the reciprocal changes in the amount of NFL normally seen *in vivo* (Fliegner et al., 1990). Chapter 2 further indicated that nIF protein subunits follow similar disassembly kinetics upon okadaic acid treatment and the extensive co-immunoprecipitation of the various subunits implied that these five proteins were associated within small oligomers likely to reflect their native

associations. Collectively, these results indicated that DRG nIFs form a highly integrated network.

Okadaic acid-induced oligomers were further characterized by BN-PAGE, a previously described, high resolution non-denaturing gel system (Schägger et al., 1994) (data not shown). Some of the smaller okadaic acid-induced species characterized by this technique appeared to consist of NFL and NFH or NFL and NFM heteromers, although the inherently ambiguous nature of whole cell extracts left some doubt as to the exact composition of these protein complexes. In addition, similar-appearing oligomers could also be generated by solubilizing nIF cytoskeletal extracts with 2 M urea and including that amount of urea within the BN-PAGE gels. The okadaic acid paradigm was thus abandoned in favor of a reassembly-type approach using purified nIF proteins.

In Chapter 3, partial nIF reassembly combined with BN-PAGE/2 M urea analysis demonstrated that NFL can dimerize with the other four nIF protein subunits and that α-internexin and peripherin can also form heterodimers with NFH or NFM. Such dimers were considered valid assembly intermediates, based on the fact that NF subunits in 2 M urea dialysed against physiological buffers assemble into normal-appearing NFs (Cohlberg et al., 1995). Consistent with their limited ability to self assemble *in vivo* or *in vitro* (reviewed in Lee and Cleveland, 1996), NFH and NFM did not show evidence of homodimer formation or heterodimer formation with each other. This further indicated that the dimer interactions that did take place were specific and were not a fortuitous consequence of simply having α-helical rod domains present on the proteins. This study constituted the first direct biochemical demonstration of heterodimeric interactions of nIF subunits.

In chapter 4, the BN-PAGE/urea system was further modified to include a lateral urea gradient which was used to assess the relative stability of NFL-containing NF protein dimers. These dimers were shown to be destabilized by a mutation in NFL suspected to affect NF assembly *in vivo* (Fuchs and Coulombe, 1992; Lee et al., 1994) and shown to cause motor neuron

degeneration reminiscent of ALS in transgenic mice (Lee et al., 1994). Furthermore, NFL extracted from spinal cord behaved differently from bacterially expressed NFL, implying that posttranslational modifications present on the spinal cord material but absent in the bacterial version could modulate the assembly properties of NFL at the dimer level. This constituted the first biochemical assessment of NF protein dimer stability and the combination of BN-PAGE with a lateral urea gradient constituted a novel appraoch for such studies.

The fact that NFH and NFM both form heterodimers with NFL while barely interacting with themselves or with each other strongly suggests that heterodimer formation is the favoured path for incorporating these proteins in growing NFs. This notion is fully consistent with the finding that NFH and NFM require the presence of NFL for proper assembly *in vitro* or *in vivo* (reviewed in Lee and Cleveland, 1996). It further suggests a mechanism whereby an excessive amount of NFH or NFM could lead to NF accumulation *in vivo*, as was observed in various transgenic mice overexpressing these proteins (Côté et al., 1993; Vickers et al., 1995). Thus, if NFL was to become substoichiometric, one would predict an increase in NF heterodimer formation at the expense of NFL homodimerization and the surplus NFL/NFH or NFL/NFM heterodimers would accumulate. To that end, the available data indicate that NF accumulations in transgenic mice that overexpress NFH (Côté et al., 1993) or NFM (Vickers et al., 1995) do contain NFL. However, mAbs used to detect the overexpressed protein do not distinguish well between NFH and NFM, and it is unclear at this time whether NF accumulations in these animals contain only the overexpressed subunit along with NFL, or whether all three proteins are present (Côté et al., 1993; Vickers et al., 1995).

The apparent effect of posttranslational modifications on NFL-containing dimer stability raises intriguing questions. Unlike the head and tail domains, the rod domain of NF proteins does not appear to contain any posttranslational modifications (Steinert et al., 1982). Thus, one may conclude that the head and/or tail domain(s) can affect NF protein associations at the level of the

coiled-coil. A similar conclusion was drawn from experiments carried out in the yeast two-hybrid system where full length NFH and NFM did not interact with each other while their isolated rod domains did (Carpenter and Ip, 1996). Although the mechanism of coiled-coil formation has been extensively studied, modulation of such interactions by protein domains lacking in α-helical and heptad repeat content has not yet been described. It will be of interest to test posttranslational modifications suspected of affecting NF assembly to delineate and characterize the regions responsible for the modulating effect observed on dimer formation.

About twenty years have passed since neurobiologists started investigating the mechanisms of nIF protein assembly. The work presented here has contributed to the body of knowledge pertaining to this area of research. As for the many more queries that remain, one may anticipate that the next twenty years will prove even more fruitful and interesting than the last.

REFERENCES

Carden, M.J., Trojanowski, J.Q., Schlaepfer, W.W. and Lee, V.M.-Y. (1987) Two stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J. Neurosci.* **7**, 3489-3504.

Carpenter, D.A. and Ip, W. (1996) Neurofilament triplet protein interactions: evidence for the preferred formation of NFL containing dimers and a putative function for the end domains. *J. Cell Science* **109**, 2493-2498.

Cohlberg, J.A., Hajarian, H., Tran, T., Alipourjeddi P., and Noveen A. (1995) Neurofilament protein heterotetramers as assembly intermediates. *J. Biol. Chem.* **270**, 9334-9339.

Côté, F., Collard, J.-F. and Julien, J.-P. (1993) Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. *Cell* **73**, 35-46.

Escurat, M., Djabali, K., Gumpel, M., Gros, F. and Portier, M.-M. (1990) Differential expression of two neuronal intermediate filament proteins, peripherin and the low molecular mass neurofilament protein (NF-L), during the development of the rat. *J.Neurosci.* **10**, 764-784.

Fliegner, K.H., Ching, G.Y. and Liem, R.K.H (1990) The predicted amino acid sequence of α-internexin is that of a novel neuronal intermediate filament protein. *EMBO J.* **9**, 749-755.

Fliegner, K.H., Kaplan, M.P., Wood, T.L., Pintar, J.E. and Liem, R.K.H. (1994) Expression of the gene for the intermediate filament protein α-internexin coincides with the onset of neuronal differentiation in the developing rat nervous system. *J. Comp. Neurol.* **342**, 161-173.

Lee, M.K. and Cleveland, D.W. (1996) Neuronal intermediate filaments. *Annu. Rev. Neurosci.* **19**, 187-217.

Lee, M.K., Marszalek, J.R. and Cleveland, D.W. (1994) A mutant neurofilament subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. *Neuron* **13**, 975-988.

Lindenbaum, M.H., Carbonetto, S., Grosveld, F., Flavell, D., and Mushynski, W.E. (1988) Transcriptional and post-transcriptional effects of nerve growth factor on expression of the three neurofilament subunits in PC12 cells. *J. Biol. Chem.* **263**, 5662-5667.

Sacher, M.G., Athlan, E.S. and Mushynski, W.E. (1992) Okadaic acid induces the rapid and reversible disruption of the neurofilament network in rat dorsal root ganglion neurons. *Biochem. Biophys. Res. Commun.* **186**, 524-530.

Schägger, H., Cramer, W.A., and von Jagow, G. (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Analyt. Biochem.* **217**, 220-230.

Steinert, P.M., Wantz, M.L. and Idler, W.W. (1982) O-phosphoserine content of intermediate filament subunits. *Biochemistry* **21**, 177-183.

Vickers, J.C., Lazzarini, R.A., Riederer, B.M. and Morrison, J.H. (1995) Intraperikaryal neurofilamentous accumulations in a subset of retinal ganglion cells in aged mice that express a human neurofilament gene. *Exper. Neurol.* **136**, 266-269.

ORIGINAL CONTRIBUTIONS TO SCIENTIFIC KNOWLEDGE

Chapter 2:

1) Established that the five nIF proteins NFL, NFM, NFH, α-internexin and peripherin are co-expressed in cultured DRG neurons from E15 rat embryos.
2) Documented the developmental expression profile of the five nIF proteins in cultured DRG neurons.
3) Demonstrated, through co-immunoprecipitation analysis, that the five nIF proteins form a highly integrated network in cultured DRG neurons.

Chapter 3:

1) Demonstrated that NFL, α-internexin and peripherin can form homodimers upon dialysis against buffered 2 M urea.
2) Demonstrated that with the exception of NFH/NFM, which remain monomeric, and α-internexin/peripherin, which could not be characterized, all pairwise combinations of nIF proteins result in heterodimer formation.
3) Provided a novel approach to the study of nIF protein dimers.
4) Provided the first direct biochemical demonstration of dimers formed by nIF proteins.

Chapter 4:

1) Revealed that posttranslational modifications present on spinal cord NFL and presumably absent on bacterially expressed NFL, reduced the stability of NFL-containing homodimers without significantly affecting the stability of dimers formed with NFH or NFM.
2) Indicated that a mutation in NFL that affected NF organization in transgenic mice reduced the stability of all NF protein dimers formed with this protein.
3) Provided a novel approach to evaluate the stability of nIF protein dimers.

APPENDIX

REPRINTS OF THE PUBLISHED VERSIONS OF

CHAPTERS 2 AND 3

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Heterodimeric Associations between Neuronal Intermediate Filament Proteins*

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Formation of protein dimers involving α-internexin, peripherin, and the neurofilament (NF) proteins NFH, NFM, and NFL was investigated by partial renaturation of various combinations of individually purified subunits in buffered 2 M urea. Oligomers that were formed were resolved by "blue" native electrophoresis (Schägger, H., Cramer, W. A., and von Jagow, G. (1994) *Anal. Biochem.* 217, 220–230) modified to include urea in the polyacrylamide gels. Combining this method with Western blot analysis, disulfide cross-linking, and SDS-polyacrylamide gel electrophoresis in the second dimension showed that NFL readily forms significant amounts of heterodimer with NFH, NFM, α-internexin, or peripherin in the presence of 2 M urea. α-Internexin and peripherin also formed heterodimers with NFH or NFM under these conditions. The modified version of blue native gel electrophoresis described here may be useful in monitoring the impact of post-translational modifications and mutations on the dimerization of intermediate filament proteins.

The cytoskeleton of most eukaryotic cells includes an intermediate filament (IF)[1] network assembled from protein subunits expressed in a cell-specific manner (see Ref. 1 for a review). IF proteins belong to a large family of related polypeptides that share a characteristic tripartite domain organization consisting of a conserved ~310-amino acid-long α-helical "rod" domain flanked by amino-terminal "head" and carboxyl-terminal "tail" domains of more heterogeneous length and composition (see Ref. 2 for a review). The first step in IF assembly involves dimerization of two identical subunits, as seen with vimentin and desmin (3), or two different subunits, as in the case of keratins (4, 5). This initial dimerization step produces an unstaggered parallel coiled-coil species (1) that can be chemically cross-linked with the copper phenanthroline (CuP_2) reagent in cases where the subunits contain a cysteine residue located in the rod domain (3).

Neuronal cells from cultured embryonic rat dorsal root ganglia (DRGs) are unusual in that they contain at least five different developmentally regulated and co-assembled neuronal IF (nIF) subunits (6). They include α-internexin (7), peripherin (8), and the neurofilament (NF) triplet proteins, which consist of the heavy (NFH), mid-sized (NFM), and low (NFL) molecular mass subunits (9). Among these five nIF proteins, only NFL has been purified and chemically cross-linked as a dimer (3), although disulfide-linked peripherin dimers that occur naturally *in vivo* have been reported (10). NFs appear to be obligate heteropolymers *in vivo* (11, 12), and individual NF subunits can also be incorporated into an α-internexin (12), peripherin (13), or vimentin (14, 15) network.

Several lines of evidence support or suggest the existence of NFH/NFL or NFM/NFL heterodimers as the smallest heteromeric unit of NF assembly. For instance, cross-linking analysis of native or partially disassembled NFs revealed that NFM could be cross-linked to NFL through cysteine residues located in the rod domain, although the approach that was used could not differentiate intra- from inter-coiled-coil dimer cross-linking (16). Furthermore, Mulligan *et al.* (17) concluded that the existence of NFH/NFL and NFM/NFL heterodimers could best reconcile their immunoelectron microscopic data with the available literature (see discussion in Ref. 17). Finally, NFH and NFM were found to interact more strongly with NFL than with themselves or with each other in a yeast two-hybrid system paradigm, suggesting that heterodimer formation with NFL is favored over NFH and/or NFM oligomerization (18, 19).

To date, however, the smallest NF hetero-oligomers examined biochemically were reported to be heterotetramers containing equimolar amounts of NFH or NFM with NFL. However, it could not be determined whether these were assembled from individual homo- or heterodimers (20). Moreover, Triton-soluble hetero-oligomers of α-internexin, peripherin, and the NF subunits have been reported, but their precise composition also remains unknown (6). Thus, the precise nature of the smallest hetero-oligomers involving the various nIF proteins is still elusive, due largely to the limitations of experimental approaches capable of resolving and characterizing these small *in vivo* or *in vitro* assembly intermediates.

A novel "blue" native polyacrylamide gel electrophoresis (BN-PAGE) system has recently been described in which Coomassie Blue G-250 is used to confer a negative charge to protein complexes, allowing them to be separated on the basis of their molecular mass while preserving their native associations (21). Initial studies conducted in our laboratory showed that BN-PAGE, rendered "semi-native" by inclusion of 2 M urea, resolved nIF proteins into what appeared to be heterocomplexes. Further studies of nIF protein complexes were initiated in which various combinations of individually purified denatured subunits were assembled by dialysis against buffered 2 M urea. Subsequent analysis of assembled protein complexes by 2 M urea-BN-PAGE, together with other techniques, demonstrated that NFL forms heterodimers with NFH or NFM and

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[1] The abbreviations used are: IF, intermediate filament; nIF, neuronal intermediate filament; CuP_2, copper phenanthroline; DRG, dorsal root ganglion; NF, neurofilament; NFH, NFM, and NFL, heavy, mid-sized, and low molecular mass neurofilament subunits, respectively; BN-PAGE, blue native polyacrylamide gel electrophoresis; DTT, dithiothreitol; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PVDF, polyvinylidene fluoride; mAb, monoclonal antibody.

that α-internexin and peripherin can form heterodimers with the individual NF subunits. These results indicate that previously reported interactions between the various nIF proteins (6) may take place at the level of heterodimer formation.

EXPERIMENTAL PROCEDURES

Preparation of Cytoskeletal Extracts—13,000 × *g* insoluble cytoskeletal extracts of embryonic cultured DRG neurons and PC12 cells were prepared essentially as described (6).

Purification of Neuronal Intermediate Filament Proteins—NFH, NFM, NFL, and α-internexin were isolated as NFs from rat spinal cord as described (22), whereas peripherin was from Triton X-100-insoluble PC12 cell extracts. These preparations were solubilized and boiled for 5 min in SDS sample buffer (2% SDS, 62.5 mM Tris (pH 6.8), 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol) (23) and separated by discontinuous SDS-PAGE in the presence of 15 mM thioglycolic acid to prevent oxidation of the cysteine residues. The proteins of interest were visualized, excised, and electroeluted as described previously (22). The electroeluted material was recovered, and solid urea was added to a concentration of 2 M. These SDS-containing solutions were then applied to individual columns of Extractigel detergent-removing matrix (Pierce) equilibrated with a degassed buffer containing 2 M urea, 50 mM Tris (pH 9.0), and 5 mM DTT. Following elution with the same buffer, an aliquot was removed for quantification and verification of protein purity and for SDS assay to ensure that the detergent had been removed (24). The protein solutions were dialyzed against a buffer containing 6 M guanidine hydrochloride, 50 mM BisTris (pH 7.0), and 5 mM DTT by repeated centrifugations in a Centricon dialysis spin column (Amicon, Montreal, Canada), aliquoted, and stored at −80 °C until further use. Proteins quantified by the bicinchoninic acid method (Sigma) were purified in the absence of reducing agents, and the SDS removal protocol was replaced with dialysis against 5 mM NaCl and 0.01% SDS in the Centricon spin columns. This permitted the removal of Tris, glycine, DTT, and excess SDS, which may interfere with the protein assay.

Cell Culture—Embryonic rat DRGs were dissected, dispersed, and maintained in defined medium as described previously (6, 25).

Partial Assembly of nIF Proteins—The individually purified nIF proteins were mixed (final concentration of 0.1–1.0 mM) and dialyzed in a Microdialyzer apparatus (Pierce) at room temperature for 2.5 h against two to three changes of an extensively degassed buffer containing 50 mM BisTris (pH 7.0) and urea at the concentrations specified in the figure legends.

Disulfide Cross-linking of nIF Protein Complexes—Dialyzed samples of nIF protein complexes were cross-linked through their cysteine residues by adding CuP_2 (3, 16, 26) from a freshly prepared concentrated stock solution to a final concentration of 15 mM. Cross-linking was carried out for 20 min at room temperature and stopped by adding EDTA to 5 mM and iodoacetamide to 50 mM (modified from Refs. 3, 16, and 26).

Analysis of nIF Protein Complexes by BN-PAGE—nIF protein complexes were resolved by BN-PAGE as described (21) with the following modifications. Stacking gels were omitted, and aminocaproic acid in the gels was replaced with 2 M urea. 5% glycerol was added to gel solutions used to pour gradient gels. The upper tank buffer contained 0.01% Coomassie Blue G-250; the 9-fold concentrated loading buffer contained 50 mM BisTris (pH 7.0), 2 M urea, 2.5% Coomassie Blue G-250, and 50% glycerol. Gels were cast in a Bio-Rad minigel apparatus and electrophoresed at 100 V. Dissociation of assembled protein complexes was achieved by heating the samples to 56 °C for 4 min immediately prior to electrophoresis, which effectively prevented reassembly. Dissociation of disulfide-linked protein complexes was achieved by including 10 mM DTT in the heated samples. For second dimension gel electrophoresis, lanes were cut out of the gels after BN-PAGE and incubated in 1.5-fold concentrated SDS sample buffer with or without 5% β-mercaptoethanol as described in the figure legends.

SDS-Polyacrylamide Gel Electrophoresis—Discontinuous gel electrophoresis of the samples on 4.5 or 6% SDS-polyacrylamide gels was performed as described (23). Electrophoresis on 4% Weber and Osborn-type gels (27) was performed essentially as described (28), except that regular Tris-containing SDS sample buffer (see above) was used for dilution of samples.

Western Blot Analysis—After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Montreal) as described (6). After electroblotting of proteins resolved by BN-PAGE, PVDF membranes were rinsed in methanol to remove Coomassie Blue. Proteins were detected with specific monoclonal antibodies (mAbs) against NFL (NR4), NFM (NN18), and NFH (N52) (Sigma) and against peripherin (mAb1527) and α-internexin (mAb1525) (Chemicon International, Inc., Temecula, CA). Probing with mAbs, erasure of the blot for subsequent reprobing, and visualization by enhanced chemiluminescence were conducted according to instructions provided by the manufacturer (NEN, Mississauga, Canada).

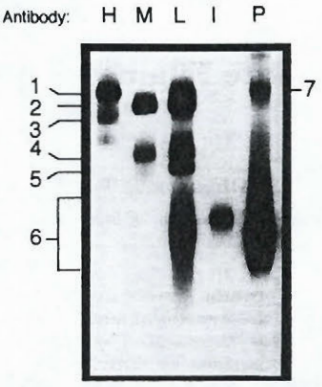

FIG. 1. **Western blot analysis of DRG cytoskeletal extracts.** Cytoskeletal pellets were purified from the axonal compartment of localized rat DRG cultures, extracted with buffered 2 M urea, resolved by 2 M urea-BN-PAGE on a 4–8% polyacrylamide gradient gel, and electroblotted onto a PVDF membrane. Strips were then cut out and reacted with mAbs against the individual nIF proteins as indicated at the top. *H*, *M*, *L*, *I*, and *P* are strips reacted with mAbs against NFH, NFM, NFL, α-internexin, and peripherin, respectively. The major protein complexes detected are numbered *1–7* and are discussed further under "Results."

Coomassie Blue Staining and Quantification of Proteins—Polyacrylamide gels were fixed for 10–20 min in destaining solution (10% isopropyl alcohol and 10% acetic acid), stained overnight in Coomassie Blue solution (0.1% Coomassie Blue R-250, 25% isopropyl alcohol, and 10% acetic acid) (29), and extensively destained against several changes of destaining solution over a 24-h period. Stained gels containing standardized amounts of rat NF proteins resolved alongside unknown samples were scanned and quantified with a BioImager apparatus (Millipore, Montreal). Subunit ratios were calculated using the following molecular masses for the rat NF subunits: NFL, 62 kDa (14); NFM, 95 kDa (30); and NFH, 115 kDa (31).

RESULTS

nIF Proteins from Cultured DRG Neurons Resolved by 2 M Urea-BN-PAGE Show Evidence of Heteromeric Associations—We previously reported that α-internexin, peripherin, and the NF triplet proteins in cultured DRG neurons were associated (6), but the methods used could not accurately determine the precise oligomeric arrangement of these proteins. Further studies in our laboratory showed that nIF extracts resolved by BN-PAGE in the presence of increasing amounts of urea contained heterocomplexes of nIF proteins. In Fig. 1, a DRG cytoskeletal pellet extracted with 2 M urea was electrophoresed using a semi-native version of BN-PAGE, which included 2 M urea in the gel. Development of the resulting Western blots with mAbs against the individual nIF proteins showed seven major bands, some of which were immunoreactive with two of the mAbs. Based on the staining intensity and the relative mobilities of the various species, protein complexes 1 and 2 appeared to be hetero-oligomers of NFL with NFH and NFM, respectively, whereas complex 4 in the anti-NFL lane appeared to be an NFL dimer. Complex 3 in the anti-NFH lane was likely the hypophosphorylated NFH-containing equivalent of complex 1 since it was much more prominent in extracts from the cell body-enriched fraction obtained from localized DRG cultures (data not shown), which we have shown to contain mostly this form of NFH (6). The broad region designated as *6* contained species staining with mAbs against NFL, α-internexin, and peripherin, suggesting the presence of homo- and/or

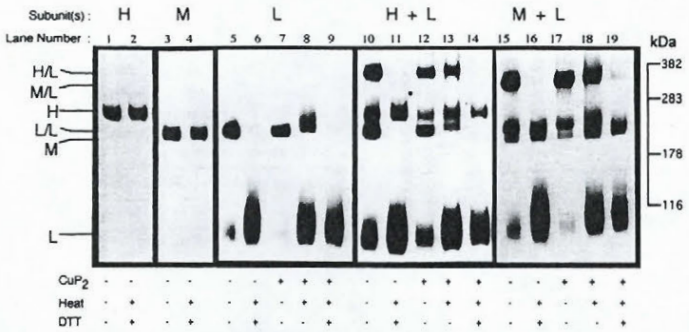

FIG. 2. **Western blot analysis of associations between purified NF subunits following dialysis against buffered 2 M urea.** NF subunits were individually purified; mixed as indicated at the top of each panel; dialyzed against buffered 2 M urea; and left untreated or treated with CuP_2, heating to 56 °C, and/or 10 mM DTT as indicated at the bottom of each lane. Samples were then resolved by BN-PAGE in 2 M urea on a 4–8% polyacrylamide gradient gel. The Western blot was developed with a mixture of mAbs against the individual NF subunits. *H*, *M*, and *L* denote the monomeric NF subunits NFH, NFM, and NFL, respectively. *H/L*, *M/L*, and *L/L* are the dimeric species containing the indicated NF subunits.

heterodimers. Complex 4 in the anti-NFM lane, complex 5 in the anti-NFL lane, and complex 7 in the anti-peripherin lane could not be identified unambiguously. Initial cross-linking studies with further resolution in a second SDS-PAGE dimension appeared to confirm some of the associations described above, and further studies were undertaken to fully characterize these and other small nIF protein complexes. This was achieved by mixing various combinations of individually purified nIF subunits in the presence of 6 M guanidine hydrochloride, dialyzing against buffered 2 M urea, and resolving the resulting complexes on semi-native BN-PAGE gels prepared with the same buffer as the dialysis solutions.

Purified NFL Forms Heterodimers with NFH and NFM —As shown in Fig. 2 (*lanes 1* and *3*), purified NFH and NFM migrated on 2 M urea-BN-PAGE as single bands with apparent molecular masses of 260 and 214 kDa, respectively. Heating to 56 °C prior to electrophoresis did not affect their mobility (*lanes 2* and *4*), indicating that NFH and NFM migrated as monomers in this gel system with anomalously high apparent molecular masses, as is also observed on SDS-PAGE (32). Gradual increases in the gel urea concentration to 8 M failed to produce smaller species, confirming the identity of the NFH and NFM bands as monomers (data not shown).

NFL dialyzed and resolved on 2 M urea-BN-PAGE (Fig. 2, *lane 5*) yielded two bands with apparent molecular masses of 226 and 94 kDa. Heating the dialyzed NFL to 56 °C prior to electrophoresis (*lane 6*) converted almost all of the larger species to the more rapidly migrating form. Disulfide cross-linking of the NFL homo-oligomer through the unique cysteine residue in the α-helical domain (14) using CuP_2 (3) did not alter the migration pattern (*lane 7*) and effectively protected a significant portion of the NFL 226-kDa species from heat-induced conversion to the 94-kDa species (*lane 8*), indicating that these were homodimers and monomers, respectively. The CuP_2-induced cross-links could be broken by including 10 mM DTT in the heated samples (*lane 9*), as would be expected for disulfide bonds.

When mixtures of NFL with NFH or NFM were analyzed by 2 M urea-BN-PAGE, the bands previously identified in the lanes containing these proteins alone were all present. In addition, new complexes appeared with apparent molecular masses of 360 kDa for the NFH/NFL mixture (Fig. 2, *lane 10*) and 328 kDa for the NFM/NFL mixture (*lane 15*), indicating the formation of NFH/NFL and NFM/NFL hetero-oligomers under these conditions. As was seen with the NFL homodimer, these hetero-oligomers were heat-labile (*lanes 11* and *16*); they could be stabilized by CuP_2 cross-linking (*lanes 13* and *18*); and the disulfide cross-links were reduced with DTT (*lanes 14* and *19*).

Given the apparent anomalous migration of nIF proteins in this gel system and because both NFH and NFM each contain more than one cysteine residue (30, 31), two complementary approaches were used to confirm that the hetero-oligomers formed between NFL and NFH or NFM were heterodimers. In Fig. 3, triplicate samples of NFH/NFL (*panel A*) and NFM/NFL (*panel B*) hetero-oligomers were prepared and resolved essentially as described for Fig. 2. The large amount of material used allowed direct visualization of all protein species in the first (2 M urea-BN-PAGE) dimension as they appeared as Coomassie Blue G-250-stained bands against a lighter blue background. The protein bands were excised, briefly equilibrated with SDS sample buffer, resolved in the second dimension by discontinuous SDS-PAGE alongside standardized mixtures of NF subunits, visualized by Coomassie Blue R-250 staining, and quantified with the BioImager apparatus. As shown in Fig. 3, the NFH/NFL and NFM/NFL hetero-oligomers purified by BN-PAGE in the first dimension broke down in the second SDS-containing dimension to give essentially equimolar amounts of NFH and NFL (ratio = 1.16 ± 0.02) or NFM and NFL (ratio = 1.06 ± 0.02) (mean ± S.D.). These results clearly demonstrate that the observed hetero-oligomers are equimolar assemblies of their NF subunit constituents.

In Fig. 4, NFH/NFL (*panel A*) and NFM/NFL (*panel B*) hetero-oligomers were formed, cross-linked, and resolved by 2 M urea-BN-PAGE essentially as described for Fig. 2. Some lanes were blotted onto a PVDF membrane and reacted with the anti-NF mAbs to reveal the positions of the various protein complexes in the first dimension as indicated at the top of Fig 4. Other lanes were cut out of the gels, equilibrated with SDS sample buffer without reducing agent, and electrophoresed in the second dimension on a Weber and Osborn-type gel (27), which provided a better estimate of the molecular mass of cross-linked proteins than was obtained with Laemmli-type gels (data not shown), as previously reported (3, 16, 28, 33, 34). Following transfer to a PVDF membrane, the blots were probed with an anti-NFL mAb (Fig. 4, *lower portions* of *panels A* and *B*), erased, and reprobed with an anti-NFH mAb (*upper portion* of *panel A*) or an anti-NFM mAb (*upper portion* of *panel B*). The cross-linked NFH/NFL hetero-oligomer seen in the first (2 M urea-BN-PAGE) dimension (*H/L*) resolved into three bands in the second SDS-PAGE dimension. The spot labeled *H/L* in the second dimension reacted with both anti-NFH and anti-NFL mAbs (*upper* and *lower portions* of *panel A*, respectively), confirming its heteromeric character. Non-cross-linked NFH (*H*)

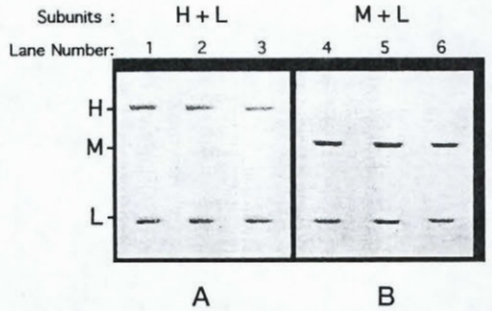

FIG. 3. **Determination of NF subunit stoichiometries in hetero-oligomers by two-dimensional gel electrophoretic analysis.** Individually purifed NFH and NFL (*panel A*) or NFM and NFL (*panel B*) were mixed, dialyzed against buffered 2 M urea, and resolved by 2 M urea-BN-PAGE on a 4–8% polyacrylamide gradient gel. The putative heterodimeric species were located, and the protein bands were cut out, briefly equilibrated with SDS sample buffer, layered on a 6% polyacrylamide gel, resolved into monomeric constituents by discontinuous SDS-PAGE (23), and stained with Coomassie Blue R-250 as described under "Experimental Procedures." *H*, *M*, and *L* denote the monomeric NF subunits NFH, NFM, and NFL, respectively.

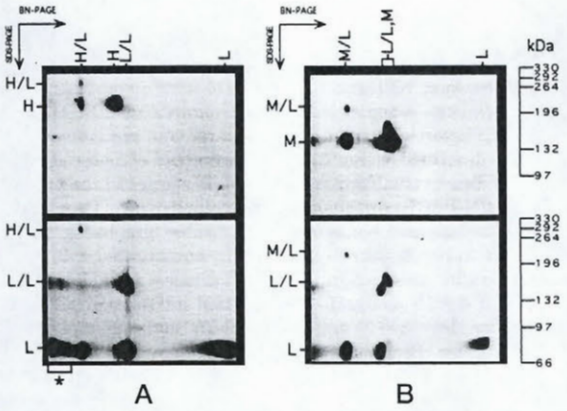

FIG. 4. **Two-dimensional gel electrophoretic determination of the relative molecular masses of cross-linked NF hetero-oligomers.** Individually purifed NFH and NFL (*panel A*) or NFM and NFL (*panel B*) were mixed, dialyzed against buffered 2 M urea, cross-linked with CuP_2 as described under "Experimental Procedures," and resolved by 2 M urea-BN-PAGE on a 4–8% polyacrylamide gradient gel. Replicate lanes were immunoblotted to identify the species indicated at the top of each panel. Other lanes were briefly equilibrated with SDS sample buffer (without reducing agent), layered on a 4% polyacrylamide Weber and Osborn-type gel (27), and resolved alongside purified NF subunits and molecular mass markers covering the 66–487-kDa range. The blots were probed with an anti-NFL mAb (*lower portions* of *panels A* and *B*), erased, and reprobed with anti-NFH and anti-NFM mAbs (*upper portions* of *panels A* and *B*, respectively). *H*, *M*, and *L* denote the monomeric NF subunits NFH, NFM, and NFL, respectively. *H/L*, *M/L*, and *L/L* are the dimeric species containing the indicated NF subunits.

and NFL (*L*) were also present in this second dimension, as could be expected from examining the heated cross-linked samples in Fig. 2 (*lanes 13* and *18*). Homodimeric NFL from the cross-linked sample seen in the first dimension (*L/L*) gave rise to some cross-linked (*L/L*) and monomeric (*L*) NFL in the second dimension, whereas the NFH and NFL species that were monomeric in the first dimension (*H* and *L*) remained so in the second dimension (*H* and *L*). Similar results were obtained for the NFM/NFL mixture (*panel B*), although cross-linked dimeric NFL (*L/L*) could not be adequately resolved from monomeric NFM (*M*), apparently due to upward streaking of the latter. It was also observed that the relative amount of cross-linked hetero-oligomer was lower on the Weber and Osborn-type gels than would be predicted from the data in Fig. 2 (*e.g. lanes 13* and *18*). This was a reproducible yet unexplained limitation of this gel system that was not observed when second dimension electrophoresis was carried out in a discontinuous SDS-PAGE system (data not shown). The apparent molecular masses of the various species detected were determined and are listed in Table I. The values for cross-linked NFH/NFL, NFM/NFL, and NFL/NFL were within 3% of the summed molecular masses of their monomeric constituents, thereby confirming their dimeric character. Taken together, the results in Figs. 2–4 and in Table I conclusively demonstrate that, under the conditions described here, NFH/NFL and NFM/NFL heterodimers and NFL/NFL homodimers are formed and can be resolved by 2 M urea-BN-PAGE.

TABLE I

Relative molecular masses of the various species resolved by two-dimensional electrophoresis after cross-linking of NFH/NFL and NFM/NFL mixtures

The samples were cross-linked and resolved by BN-PAGE followed by SDS-PAGE as described under "Experimental Procedures" and in the legend to Fig. 4. H, M, and L denote the NF subunits NFH, NFM, and NFL, respectively.

Protein species	Relative molecular mass	Predicted molecular mass
	kDa	*kDa*
H·L complex	308	317
M·L complex	239	246
L·L complex	158	156
H (monomeric)	239	
M (monomeric)	168	
L (monomeric)	78	

α-Internexin and Peripherin Form Heterodimers with NF Subunits—In Fig. 5, α-internexin and peripherin were tested for their ability to dimerize alone or in combination with other nIF proteins. The experiments were carried out essentially as described for Fig. 2, except that cross-linking and heating of some species was omitted. Some of the previously characterized NF species are shown again to provide useful molecular mass landmarks. Based on the relative mobilities of the protein complexes, both α-internexin and peripherin formed homodimers (~159 and ~143 kDa in *lanes 5* and *7*, respectively) that broke down to the monomeric species upon heat treatment (~73 and ~66 kDa in *lanes 6* and *8*, respectively). The staining intensity of the heat-treated peripherin sample was reproducibly lower than that of the unheated sample (*lanes 8* and *7*, respectively), apparently due to in-gel dimerization of the monomeric species, leading to extensive streaking and concomitant loss of signal. The significant amount of dimeric peripherin in the heated sample and the virtual absence of monomeric peripherin in all of the other peripherin-containing lanes (*lanes 7*, *9*, and *14–16*) confirm its very strong tendency to dimerize under the conditions used. The various dimeric species obtained when mixing α-internexin and peripherin (*lane 9*) could not be resolved adequately on these gels, and cross-linking experiments failed to show any heterodimeric interactions between these two proteins (data not shown). Based on the relative mobility of the protein complexes, heterodimers containing α-internexin or peripherin with NFH (~356 kDa) or NFM (~320 kDa) (*lanes 10*, *11*, *14*, and *15*, respectively) were also observed. Although the relative yields of these heterodimers were much lower than those observed for the NFH/NFL and NFM/NFL species, markedly greater amounts could be generated by dialyzing and resolving the samples in 0.05 M urea instead of 2 M urea (data not shown). It is not known why the α-internexin/NFH and α-internexin/NFM heterodimers in Fig. 5 migrated as doublets, although protein degradation seems unlikely since it was not observed in the purified subunit preparations (data not shown). Mixing α-internexin or peripherin

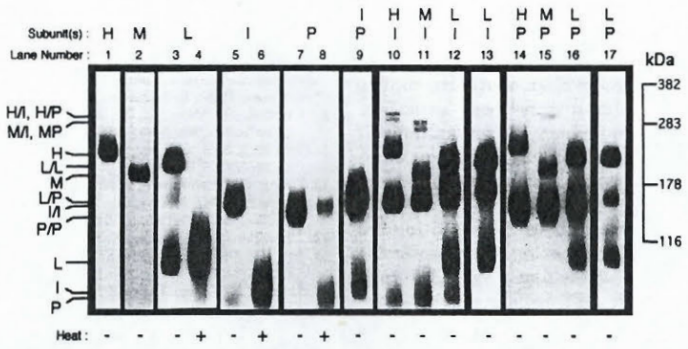

FIG. 5. **Western blot analysis of associations between purified nIF subunits following dialysis against buffered 2 M urea.** nIF subunits were individually purified, mixed as indicated at the top of each panel, and dialyzed against buffered 2 M urea, and some samples were heated to 56 °C as indicated at the bottom of each lane. Samples were then resolved by 2 M urea-BN-PAGE on a 4–8% polyacrylamide gradient gel. The Western blot was developed with a mixture of mAbs against the individual nIF subunits. *Lanes 12* and *16* were erased, reprobed with an anti-NFL mAb only, and are shown in *lanes 13* and *17*, respectively. *H*, *M*, *L*, *I*, and *P* denote the nIF monomeric subunits NFH, NFM, NFL, α-internexin, and peripherin, respectively. *L/L*, *H/I*, *M/I*, *I/I*, *H/P*, *M/P*, *L/P*, and *P/P* denote the dimeric species containing the various nIF proteins.

with NFL produced heterodimers that could not be resolved from the α-internexin and peripherin homodimers (*lanes 12* and *16*, respectively). However, erasure of the Western blot and reprobing with an anti-NFL mAb alone clearly revealed the presence of α-internexin/NFL (~177 kDa in *lane 13*) and peripherin/NFL (~167 kDa in *lane 17*) heterodimers.

DISCUSSION

We recently reported that α-internexin, peripherin, and the NF triplet proteins in cultured DRG neurons are associated (6), thereby raising questions regarding the assembly stage during which such heteromeric associations can take place. Characterization of IF protein dimers has been hampered by a lack of practical and widely accessible techniques for resolving small oligomeric assembly intermediates. We have now shown that the recently reported BN-PAGE procedure (21), modified to include urea, can provide direct and unambiguous separation of nIF protein complexes and allows multiple combinations of nIF proteins to be resolved in parallel. To this end, Fig. 2 provides direct evidence for heterodimer formation between NFL and NFH or NFM, whereas Fig. 5 further shows heterodimeric associations between α-internexin or peripherin and the individual NF subunits.

Small IF assembly intermediates have been generated by a variety of means, all revolving around the use of low ionic strength buffers, sometimes containing urea or guanidine in the 2–3 M range. Such buffers have been used either as a diluent of whole IFs (16) or as the dialysis component for limited reassembly of IF proteins from concentrated guanidine or urea solutions (3–5, 20, 35, 36). IF protein oligomers generated under these conditions have been considered to be valid assembly intermediates since addition of physiological salt concentrations coupled with removal of urea or guanidine, if required, leads to the formation of normal-appearing IFs. Moreover, reassembly of NFs (37, 38), α-internexin (7, 39), and peripherin (40) from urea-containing buffers has been well documented. More specifically, NFL and NFM have been successfully co-assembled into native-like filaments by stepwise dialysis from guanidine solution to a physiological buffer, including an intermediate step involving a buffered 2 M urea solution similar to ours (20). Taken together, these data indicate that other nIF protein oligomers formed under such conditions would be valid assembly intermediates.

A previous report described the resolution of NF subunit heterotetramers containing equimolar amounts of NFL with NFH or NFM on "native" gels containing 2 M urea (20). In contrast, our study using the BN-PAGE system revealed that dimers were the predominant species under similar conditions. It is possible that the presence of Coomassie Blue G-250 destabilizes tetrameric species, thus favoring the dimers that we observed, although such effects were not reported by Schägger *et al.* (21). Alternatively, the bovine material used by Cohlberg *et al.* (20) may form complexes that are more stable in 2 M urea than those formed by the rat nIF proteins utilized in our work. However, neither of these possibilities affects the present conclusions relating to heterodimer formation.

NFH and NFM did not interact with themselves (Figs. 2 and 5) or with each other (data not shown), consistent with the many reports indicating that NFM and NFH do not assemble or co-assemble significantly *in vitro* (37, 38, 41) or *in vivo* (11, 12) and further indicating that the interactions we did observe were not likely to be due to nonspecific interactions between related α-helical rod domains.

The first step in IF assembly involves the formation of an unstaggered parallel coiled-coil dimer (2, 42). Residues in positions *a* and *d* in the heptad repeats making up the α-helices are believed to form inward facing hydrophobic seams, whereas the other residues face outward (reviewed in Ref. 43). NFL contains a unique cysteine residue located in a *d* position within a heptad repeat in the rod domain (14). Disulfide cross-linking of NFL homodimers and other IF protein dimers sharing this conserved residue has been well documented and has provided an important argument favoring the unstaggered parallel coiled-coil model of IF homodimer structure (3, 43). However, disulfide bond formation between NFL and NFH or NFM under the conditions used here is somewhat puzzling. Indeed, the location of the closest cysteine residue in the rod domain of NFH in the linker region L2, 70 residues removed from the cysteine residue in NFL, appears to preclude an unstaggered alignment of these proteins. A similar problem involves the cysteine in NFM occupying the *a* position in a heptad, 41 residues away from the cysteine in NFL. Yet, Fig. 3 clearly demonstrated that cross-linked NFL/NFH and NFL/NFM dimers can be resolved as such in the 2 M urea-BN-PAGE dimension, raising the possibility that, in contrast to other IF proteins characterized so far (1–3, 42), NF subunits associated in a coiled-coil heterodimer may be in a staggered arrangement. This interpretation appears unlikely considering that some twisting of the helices would also be required to adequately align the cysteine residues involved in the cross-linking of NFM or NFH to NFL. A more likely explanation may be that

the 2 M urea-containing buffer used here allows for slippage and flexibility within the coiled-coil structure, which would permit interactions between cysteine residues that are widely separated in the normal unstaggered alignment. According to this view, the mobility of subunits in such loosely assembled coiled-coil dimers would still allow for more efficient intradimer cross-linking compared with cross-linking between unassembled random collision complexes, which were never observed in these experiments.

α-Internexin and peripherin were shown in Fig. 5 to form heterodimers with NFH or NFM, suggesting that co-assembly of these proteins can take place at the dimer level. The comparatively greater propensity for NFL to form heterodimers with NFH and NFM further suggests that it is the preferred partner for these proteins. However, heterodimeric association of α-internexin or peripherin with the NF subunits may be important for cytoskeletal reorganization during development and axonal regeneration. α-Internexin is the predominant nIF protein in developing central neurons (39, 44), whereas peripherin expression is up-regulated in regenerating large-caliber NF-containing peripheral axons (45). In both instances, these α-internexin- or peripherin-containing networks are presumed to confer greater plasticity as reflected in the dynamic nature of the growing axon (46). The ability of NF proteins to dimerize with α-internexin and peripherin could play an important role in allowing a gradual transition to take place from a more plastic α-internexin- or peripherin-containing network to a stable NF-enriched IF network in the mature axon (47).

The modified version of BN-PAGE described here is a widely accessible, economical, and versatile method that allows several different samples to be analyzed in parallel. Future work in our laboratory will focus on monitoring early nIF protein assembly intermediates and on assessing the effects of mutations or post-translational modifications known or suspected to affect IF assembly.

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REFERENCES

1. Fuchs, E., and Weber, K. (1994) *Annu. Rev. Biochem.* **63,** 345–382
2. Steinert, P. M., and Roop, D. R. (1988) *Annu. Rev. Biochem.* **57,** 593–625
3. Quinlan, R. A., Hatzfeld, M., and Franke, W. W. (1986) *J. Mol. Biol.* **192,** 337–349
4. Hatzfeld, M., and Weber, K. (1990) *J. Cell Biol.* **110,** 1199–1210
5. Steinert, P. M. (1990) *J. Biol. Chem.* **265,** 8766–8774
6. Athlan, E. S., Sacher, M. S., and Mushynski, W. E. (1997) *J. Neurosci. Res.* **47,** 300–310
7. Chiu, F. C., Barnes, E. A., Das, K., Haley, J., Socolow, P., Macaluso, F. P., and Fant, J. (1989) *Neuron* **2,** 1435–1445
8. Portier, M.-M., de Néchaud, B., and Gros, F. (1984) *Dev. Neurosci.* **6,** 335–344
9. Hoffman, P. N., and Lasek, R. J. (1975) *J. Cell Biol.* **66,** 351–366
10. Chadan, S., Moya, K. L., Portier, M.-M., and Filliatreau, G. (1994) *J. Neurochem.* **62,** 1894–1905
11. Lee, M. K., Xu, Z., Wong, P. C., and Cleveland, D. W. (1993) *J. Cell Biol.* **122,** 1337–1350
12. Ching, G. Y., and Liem, R. K. H. (1993) *J. Cell Biol.* **122,** 1323–1335
13. Parysek, L. M., McReynolds, M. A., Goldman, R. D., and Ley, C. A. (1991) *J. Neurosci. Res.* **30,** 80–91
14. Chin, S. S. M., and Liem, R. K. H. (1989) *Eur. J. Biochem.* **50,** 475–490
15. Sun, D., Macioce, P., Chin, S. S. M., and Liem, R. K. H. (1997) *J. Neurochem.* **68,** 917–926
16. Carden, M. J., and Eagles, P. A. M. (1986) *Biochem. J.* **234,** 587–591
17. Mulligan, L., Balin, B. J., Lee, V. M.-Y., and Ip, W. (1991) *J. Struct. Biol.* **106,** 145–160
18. Carpenter, D. A., and Ip, W. (1996) *J. Cell Sci.* **109,** 2493–2498
19. Leung, C. L., and Liem, R. K. H. (1996) *J. Biol. Chem.* **271,** 14041–14044
20. Cohlberg, J. A., Hajarian, H., Tran, T., Alipourjeddi, P., and Noveen, A. (1995) *J. Biol. Chem.* **270,** 9334–9339
21. Schägger, H., Cramer, W. A., and von Jagow, G. (1994) *Anal. Biochem.* **217,** 220–230
22. Julien, J.-P., and Mushynski, W. E. (1982) *J. Biol. Chem.* **257,** 10467–10470
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685
24. Sokoloff, R. L., and Frigon, R. P. (1981) *Anal. Biochem.* **118,** 138–141
25. Giasson, B. I., and Mushynski, W. E. (1996) *J. Biol. Chem.* **271,** 30404–30409
26. Kobashi, K. (1968) *Biochim. Biophys. Acta* **158,** 239–245
27. Weber, K., and Osborn, M. J. (1969) *J. Biol. Chem.* **244,** 4406–4412
28. Sigma (1988) *Sigma Technical Bulletin MWS-877 X,* St. Louis, MO
29. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) *Biochemistry* **10,** 2606–2617
30. Napolitano, E. W., Chin, S. S. M., Colman, D. R., and Liem, R. K. H. (1987) *J. Neurosci.* **7,** 2590–2599
31. Chin, S. S. M., and Liem, R. K. H. (1990) *J. Neurosci.* **10,** 3714–3726
32. Kaufmann, E., Geisler, N., and Weber, K. (1984) *FEBS Lett.* **170,** 81–84
33. Bragg, P. D., and Hou, C. (1975) *Arch. Biochem. Biophys.* **167,** 311–321
34. Bretscher, A., and Weber, K. (1980) *Cell* **20,** 839–847
35. Cohlberg, J. A., Hajarian, H., and Sainte-Marie, S. (1987) *J. Biol. Chem.* **262,** 17009–17015
36. Hisanaga, S., Ikai, A., and Hirokawa, N., J. (1990) *J. Mol. Biol.* **211,** 857–869
37. Zackroff, R. V., Idler, W. W., Steinert, P. M., and Goldman, R. D. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 754–757
38. Liem R. K. H., and Hutchison, S. B. (1982) *Biochemistry* **21,** 3221–3226
39. Kaplan, M. P., Chin, S. S. M., Fliegner, K. H., and Liem, R. K. H. (1990) *J. Neurosci.* **10,** 2735–2748
40. Parysek, L. M., and Goldman, R. D. (1987) *J. Neurosci.* **7,** 781–791
41. Geisler, N., and Weber, K. (1981) *J. Mol. Biol.* **151,** 565–571
42. Parry, D. A. D., Steven, A. C., and Steinert, P. M. (1985) *Biochem. Biophys. Res. Commun.* **127,** 1012–1018
43. Cohen, C., and Parry, D. A. D. (1990) *Proteins Struct. Funct. Genet.* **7,** 1–15
44. Fliegner, K. H., Kaplan, M. P., Wood, T. L., Pintar, J. E., and Liem, R. K. H. (1994) *J. Comp. Neurol.* **342,** 161–173
45. Wong, J., and Oblinger, M. M. J. (1990) *Neurosci. Res.* **27,** 332–341
46. Nixon, R. A., and Shea, T. B. (1992) *Cell Motil. Cytoskeleton* **22,** 81–91
47. Giasson, B. I., and Mushynski, W. E. (1997) *Neurosci. Lett.* **229,** 77–80

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Associations Between Intermediate Filament Proteins Expressed in Cultured Dorsal Root Ganglion Neurons

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The developmental profile of the neurofilament (NF) triplet proteins, α-internexin and peripherin in cultured dorsal root ganglion neurons from gestation day 15 rat embryos was determined by Western blot analysis. At the outset (day 0 in culture), the neurons contained mostly α-internexin. A significant increase in peripherin levels was seen at days 1–2, in the mid-sized (NFM) and low molecular weight (NFL) NF subunits at days 2–3, and in the high molecular weight (NFH) NF subunit at days 5–6. Immunofluorescence microscopy showed that the five intermediate filament proteins were co-localized in all neuronal cell bodies and neurites. Analysis of Triton X-100 extracts from okadaic acid–treated dorsal root ganglion cultures revealed that peripherin and α-internexin followed the same fragmentation pattern observed with NFs. Interactions between the various neuronal intermediate filament proteins in these extracts were assessed by immunoprecipitation under native conditions using antibodies specific for the individual proteins. Co-immunoprecipitation of NFH with NFL, NFM with NFL, NFM with α-internexin, and α-internexin with peripherin demonstrated that the intermediate filament cytoskeleton in cultured sensory neurons is a highly integrated structure. J. Neurosci. Res. 47:300–310, 1997. © 1997 Wiley-Liss, Inc.

Key words: neurofilaments; peripherin; α-internexin; okadaic acid

INTRODUCTION

Neurofilaments (NFs) were long considered to be the major type of intermediate filament (IF) expressed in mature neurons (Shaw et al., 1981; Trojanowski et al., 1986). However, neurons have more recently been shown to express two additional IF proteins, peripherin (Portier et al., 1984; Leonard et al., 1988; Parysek et al., 1988) and α-internexin (Fliegner et al., 1990). The expression of peripherin and α-internexin overlaps with that of NF proteins during development and in a subset of adult neurons (reviewed by Nixon and Shea, 1992).

Mammalian NFs are composed of three phosphoprotein subunits with apparent molecular masses of about 68 KDa (NFL), 150 KDa (NFM), and 200 KDa (NFH), as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Hoffman and Lasek, 1975; Julien and Mushynski, 1982). As members of the IF family of proteins, NF subunits display a characteristic domain organization, consisting of an amino-terminal head and a carboxy-terminal tail flanking a highly conserved α-helical rod domain (Geisler et al., 1983).

The occurrence of a rod domain in each NF subunit suggested that the individual proteins might be capable of forming filaments, since IF assembly begins with a coiled-coil dimerization step involving this domain (for a review, see Fuchs and Weber, 1994). However, only NFL is capable of forming substantial, 10-nm-wide homopolymeric filaments in vitro (Geisler and Weber, 1981), and in vivo studies have shown that NFs are obligate heteropolymers whose formation requires NFL together with NFM and/or NFH (Ching and Liem, 1993; Lee et al., 1993; Ohara et al., 1993; Nagakawa et al., 1995).

The finding that NFs are obligate heteropolymers has invited comparison with keratin IFs, which have similar properties. However, keratin IFs are stoichiometric heteteropolymers requiring the participation of an acidic and a basic type keratin in coiled-coil dimerization (Fuchs and Weber, 1994). The conditions for NF assembly are less rigid, requiring NFL together with a substoichiometric amount of either NFM or NFH (Lee et al., 1993). There is evidence that heteromeric interactions between NF subunits occur during early stages of NF assembly, at the dimer or tetramer level (Carden and Eagles, 1986; Cohlberg et al., 1995). However, the formation of NFs in cells expressing NFM or NFH at one-tenth the level of NFL (Lee et al., 1993) indicates that

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homopolymeric stretches can be accomodated within the NF structure.

NF assembly and structure may be more complex in neurons that also express peripherin and α-internexin. Transfection studies with an IF-deficient cell line have shown that α-internexin and peripherin can form homopolymeric filaments (Ching and Liem, 1993; Cui et al., 1995). α-Internexin can also co-assemble with each of the three NF subunits in such transfected cells (Ching and Liem, 1993) as well as in vitro (Balin and Miller, 1995), and peripherin was shown to co-localize with NF triplet proteins in a subset of neuronal IFs in the sciatic nerve (Parysek et al., 1991). However, little is known about the extent or nature of such interactions in neurons.

Dorsal root ganglia (DRGs) in adult rats contain two distinct types of neurons differing in size and IF content. The small neurons express peripherin, while the large neurons express NFs (Goldstein et al., 1991). On the other hand, embryonic DRGs in vivo or cultures of embryonic day 15 (E15) DRG neurons contain a single neuronal type expressing both peripherin and NFs (Goldstein et al., 1996). α-Internexin is also found in embryonic DRG neurons in vivo (Fliegner et al., 1994), although its expression declines postnatally (Chiu et al., 1989; Kaplan et al., 1990).

The co-assembly of different IF proteins is often assessed by methods such as immunofluorescence microscopy (Ching and Liem, 1993; Lee et al., 1993) or immunoelectron microscopy (Balin et al., 1991; Parysek et al., 1991; Balin and Miller, 1995), which provide little insight into the types of interactions involved. The availability of a method known to cause the fragmentation of NFs in cultured DRG neurons has enabled us to take a different approach to study interactions between the various neuronal IF proteins. Indeed, treating DRG neurons with okadaic acid (OA), a potent inhibitor of protein phosphatase-2A and protein phosphatase-1 (Cohen et al., 1990), causes NFs to fragment (Sacher et al., 1992, 1994). In this report we show that peripherin and α-internexin co-localize with NFs in cultured E15 DRG neurons and undergo OA-induced fragmentation similar to that of NFs. In such OA-treated cultures, we have characterized the associations between the various neuronal IF proteins in Triton X-100 (Triton)–soluble oligomers by immunoprecipitation analysis. Our results indicate that α-internexin is a key element in the integration of the various neuronal IF proteins through its predominant association with NFM and peripherin.

MATERIALS AND METHODS

Materials

OA was from LC Services (Woburn, MA). Specific, phosphorylation-independent monoclonal antibodies (Abs) against NFL (NR4), NFM (NN18), and NFH (N52) were from Sigma Chemical Co. (St. Louis, MO). Monoclonal Abs against peripherin (MAB1527) and α-internexin (MAB1525) were from Chemicon International, Temecula, CA), while the one against vimentin (V9) was from Boehringer Mannheim (Montreal, Canada). Enhanced chemiluminescence reagents were from NEN (Mississauga, Canada). Neuronal IF proteins were purified by preparative SDS-PAGE as described (Julien and Mushynski, 1982). The NF proteins and α-internexin were from adult rat spinal cord, while peripherin was from PC12 cells.

Cell Culture and Developmental Profile of Neuronal IF Proteins

Rat DRGs were dissected, dispersed, and maintained in defined medium as previously described (Sacher et al., 1992). For developmental analysis of neuronal IF protein expression, cells were plated on 12-well tissue culture dishes and allowed to attach to the collagen substrate for 1 hr. The medium was then drained and replaced, and the first plated sample (time = 0 hr) was harvested in sample buffer (2% SDS, 62.5 mM TrisHCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol) (Laemmli, 1970). An untrypsinized sample and a trypsinized but unplated sample were also kept for analysis. Standardization of the samples was achieved by harvesting in equal volumes of sample buffer and by loading identical volumes on the gels. This is a suitable method since E15 neurons are post-mitotic and do not die to any significant degree during the first 30 to 40 days in culture (E.A., unpublished observation). For separate analysis of neurites and neuronal cell bodies, the cells that would normally be used for culture in a dispersed configuration were concentrated down to a very small volume (10 μl), plated at the center of a 35-mm dish, and allowed to attach for 0.5–1.0 hr at 37°C. The dishes were then flooded with medium, and the resulting localized cultures consisted of a central neuronal cell body mass eventually surrounded by a halo of neurites.

OA Treatment and Time-Course Analysis

Twenty-five to 32-day-old cultures were treated with 1 μM OA for 0, 1, 2, and 4 hr. Neuronal cell bodies and neurites were physically separated for analysis using a punch with a diameter equal to that of the cell body mass. Samples were harvested in cytoskeleton extraction buffer (CSK buffer: 1% Triton, 100 mM NaCl, 50 mM TrisHCl, pH 7.5, 50 mM NaF, 2 mM EDTA, 2 mM levamisol, 1 mM phenylmethylsulfonyl fluoride), vortexed for 30 sec, and centrifuged at 13,000*g* for 15 min. The resulting pellets (13K pellet) were dissolved in SDS-Sample buffer, and the 13,000*g* supernatants (13K supernatant) were further centrifuged at 100,000*g* in a

Beckman airfuge for 5 min. The resulting pellets (100K pellet) and supernatants (100K supernatant) were dissolved in sample buffer or by adding one-half volume of 3× SDS-sample buffer, respectively. The 13K and 100K pellets and the 100K supernatants were in identical final volumes, and equal volumes were analyzed by SDS-PAGE and Western blotting.

Immunoprecipitation

13K supernatants from cultures treated with OA for 4 hr were immunoprecipitated for 1 hr at 4°C with one of the monoclonal Abs against either NFL, NFM, NFH, α-internexin, or peripherin bound to anti-mouse IgG1 Abs crosslinked to agarose beads (Sigma Chemical Co., St. Louis, MO). After centrifugation, the pelleted beads were washed repeatedly with CSK buffer. The immunoprecipitated proteins were solubilized by boiling for 5 min in SDS sample buffer and analyzed by SDS-PAGE and Western blotting.

SDS-PAGE and Western Blotting

Electrophoresis of the samples on 6% SDS-polyacrylamide gels was performed as described (Laemmli, 1970). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Inc.) in a buffer containing 48 mM Tris and 39 mM Glycine. The protein bands were then detected with the Abs mentioned in the figure legends and visualized by enhanced chemiluminescence as described by the manufacturer.

Immunofluorescence

Twenty-five to 32-day-old dispersed sister cultures grown on collagen-coated glass slides were rinsed with phosphate-buffered saline (PBS: NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 10 mM; KH_2PO_4, 1.8 mM; pH 7.4) and fixed with methanol for 20 min at −20°C. Following rehydration, the samples were incubated for 30 min at 37°C with blocking buffer (PBS with 10% goat serum and 0.3% Triton) and stained for 1 hr at 37°C with a cocktail containing a rabbit polyclonal anti-NFM Ab (1:100) and one of the commercial monoclonal Abs (1:100 to 1:500) against the individual neuronal IF proteins diluted in blocking buffer. The slides were then rinsed 5 to 6 times for 5 min with PBS containing 0.3% Triton and incubated with Texas red- or DTAF-conjugated species-specific secondary Abs (1:100) and rinsed under the same conditions as used with the primary Abs. Slides were then photographed using black-and-white KODAK TMAX400 and a Zeiss microscope with the camera set on automatic exposure. Typical exposures were from 15 to 25 sec for the texas-red filter and 25 to 60 seconds for the DTAF filter.

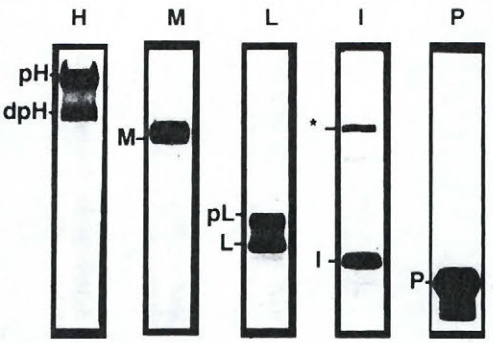

Fig. 1. Verification of the specificity of the monoclonal Abs against the individual neuronal IF proteins by Western blot analysis. DRG samples containing the various phosphovariants of NFL and NFH were resolved by SDS-PAGE, and individual lanes were developed with one of the monoclonal Abs. The letters H, M, L, I, and P at the top of the individual lanes refer to the monoclonal Abs against NFH, NFM, NFL, α-internexin, and peripherin, respectively. pH and dpH, hyper- and hypophosphorylated forms of NFH, respectively; M, NFM; pL and L, hyperphosphorylated and normal NFL, respectively; I, α-internexin; P, peripherin; *, a cross-reactive band of unknown origin.

RESULTS

The specificity of the monoclonal Abs used in this study was verified by testing them individually on a Western blot containing all of the neuronal IF proteins, including phosphovariants of NFH and NFL. The results in Figure 1 indicate that the individual Abs showed no appreciable cross-reactivity with the other IF proteins. Note that since anti-α-internexin also labeled an unknown Triton-soluble band (*) migrating just above NFM, only the bottom half of Western blots was probed with this antibody.

Available in vivo and in vitro data relating to neuronal IF expression in adult and embryonic rat DRGs (Chiu et al., 1989; Kaplan et al., 1990; Goldstein et al., 1991, 1996; Fliegner et al., 1994) suggested that cultures of E15 DRG neurons would express peripherin and α-internexin along with the NF triplet proteins. The results in Figure 2 indicate that before trypsinization, E15 DRG neurons contained mainly α-internexin with traces of peripherin and NFL (Fig. 2, lane B). Trypsinization alone had little impact on the profile of neuronal IF proteins (Fig. 2, lane T). One hour after plating, the harvested samples had lesser amounts of these proteins, due largely to the observed failure of some neurons to attach to the collagen substrate. There was an ordered increase in the levels of neuronal IF proteins. The neurons contained

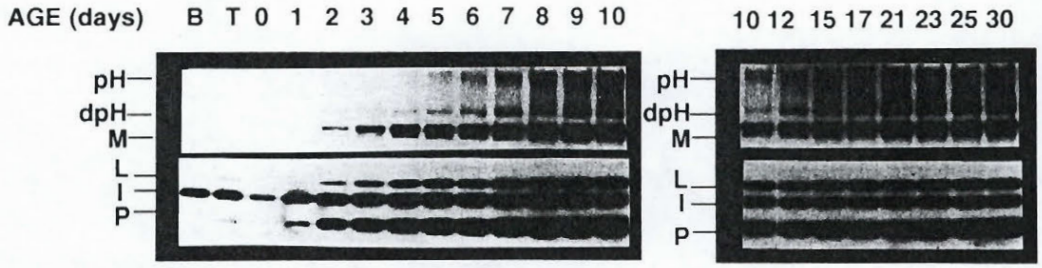

Fig. 2. Western blot analysis of the developmental profile of neuronal IF proteins in cultured E15 DRG neurons. DRGs were cultured as described in Materials and Methods and harvested in equal volumes of sample buffer at the times (in days) indicated at the top of each lane. In the panel on the right, sample volumes were one-fourth of those in the left-hand panel to permit visualization of the entire range of samples with a single film exposure. The day 10 sample was reloaded in the right panel at one-fourth the volume for comparison. The upper part of the blot was probed with monoclonal Abs against NFH and NFM, while the lower part was probed with monoclonal Abs against NFL, α-internexin, and peripherin. IF protein bands are designated as described in Figure 1. B and T, before and after trypsin, respectively.

mainly α-internexin at the outset (day 0). An increase in peripherin levels was seen at days 1–2, in NFL and NFM at days 2–3, and in NFH at days 5–6. Beyond day 17 the levels of all five IF proteins changed very gradually.

To address the question of whether the neuronal IF proteins co-localize in the same neurons, dispersed cultures grown on collagen-coated glass slides for twenty five days were double stained with a rabbit polyclonal anti-NFM Ab and a monoclonal Ab against one of the neuronal IF protein. The DTAF- and Texas red-conjugated secondary Abs showed no detectable species cross-reactivity (data not shown). The fluorescence micrographs in Figure 3 show that monoclonal Abs against the individual neuronal IF proteins each stained the same neurites (A′ to E′) and neuronal cell bodies (F′) as the polyclonal anti-NFM Ab (A to F), indicating that all neuronal IF proteins are completely co-localized in these cultured DRG neurons. However, differences in staining intensities of some neurites were observed, indicating that the relative proportions of different IF proteins in these processes can vary. Neuronal cell bodies were also stained by antibodies against all of the neuronal IF proteins; hence only the results for anti-NFM and anti-α-internexin are shown in Figure 3.

As a first step toward determining whether the various neuronal IF proteins are associated or form independent structures, we tested whether OA treatment would fragment assembled peripherin and α-internexin in the same way as it does NFs (Sacher et al., 1994). Localized DRG cultures were treated with 1 μM OA for 1–4 hr, the cell bodies and neurites were harvested separately in CSK buffer, and various fractions were obtained by differential centrifugation (see Materials and Methods). The 13K and 100K pellets and 100K supernatant were resolved by SDS-PAGE, and Western blots were probed with Abs against NFH, NFM, NFL, α-internexin, and peripherin (Fig. 4). In the untreated perikaryal sample (0 hr), most of the NFH was hypophosphorylated, while axonal NFH was exclusively hyperphosphorylated as can be expected from in vivo observations (Sternberger and Sternberger, 1983). A continuous reduction in the gel electrophoretic mobility of perikaryal NFH (dpH) was seen over the 4-hr course of OA treatment, reflecting its increased phosphorylation in the presence of the phosphatase inhibitor. The perikaryal compartment also differed from the axonal compartment in that the untreated sample contained a greater proportion of Triton-soluble NFH, NFL, and peripherin, perhaps reflecting the presence of newly synthesized, unassembled subunits. The amounts of all five neuronal IF proteins in the 100K pellet from cell bodies and neurites reached a maximum at 2 hr and then leveled off. In contrast, IF protein levels in the 100K supernatant increased continuously over the 4-hr time-course, indicating that neuronal IFs in OA-treated neurons were first fragmented into large, 13,000*g*-soluble oligomers and then underwent further fragmentation to a point where they were soluble at 100,000*g*. This sequence is essentially the reverse of that which occurs during incorporation of newly synthesized NF subunits into the cytoskeleton (Shea et al., 1988).

Estimates based on the Western blot in Figure 4 indicate that roughly two-thirds of the neuronal IF proteins ended up in the 13K supernatant after 4 hr of OA treatment. A hyperphosphorylated form of NFL (pL), with a markedly reduced mobility, appeared in cell bodies and neurites at this time and was found predominantly in the 100K supernatant.

The finding that α-internexin and peripherin were also rendered Triton soluble by OA treatment suggested that they might be coassembled with NFs. To test this possibility, Triton-soluble IF oligomers produced in DRG neurons treated with OA for 4 hr were further

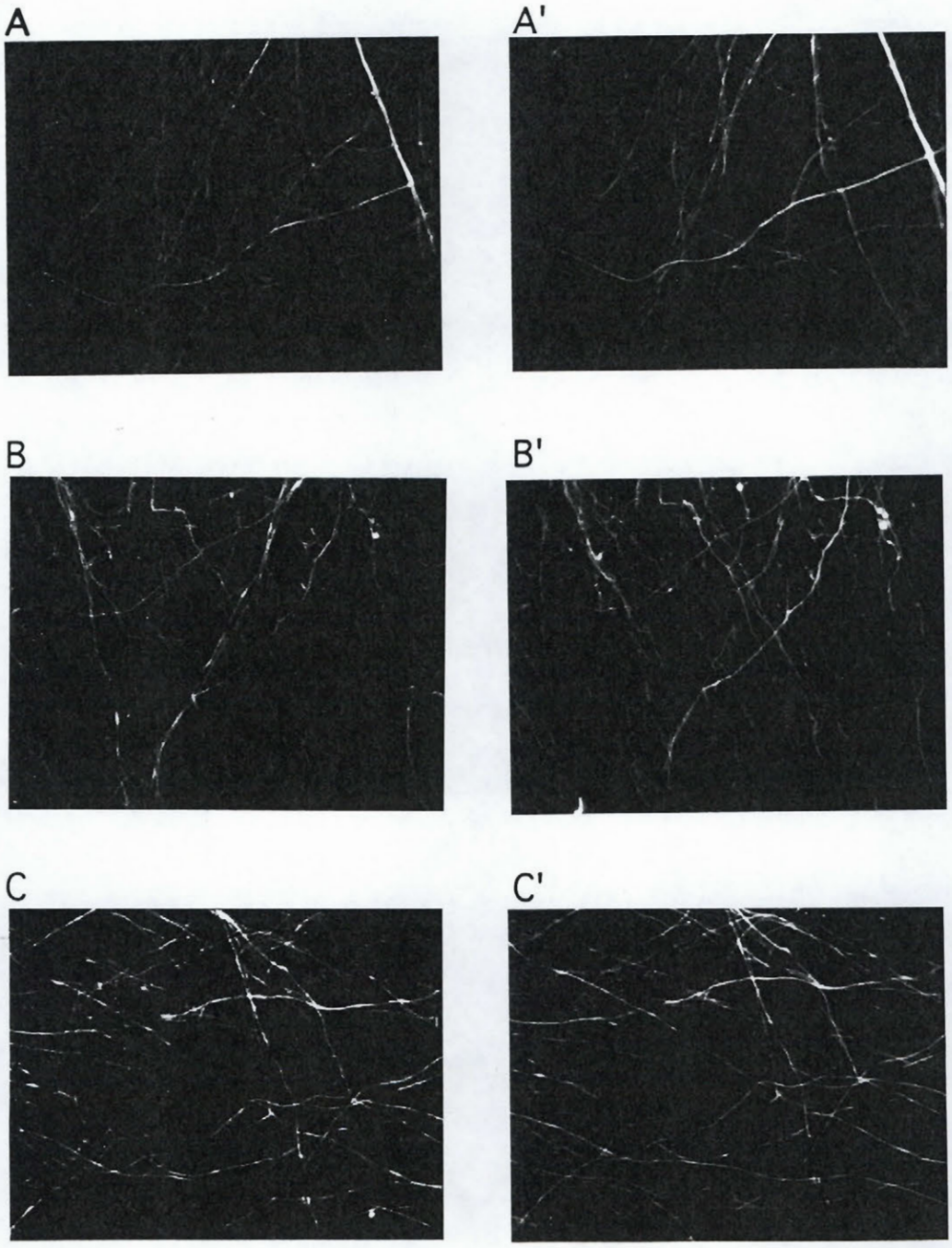

Fig. 3. Co-localization of neuronal IF proteins in cultured E15 DRG neurons by immunofluorescence microscopy. Cells were plated on collagen-coated glass slides, fixed, and stained as described in Materials and Methods. **A–F:** Sister cultures stained with a rabbit polyclonal anti-NFM Ab. **A′–F′:** Co-staining with monoclonal Abs specific for NFH (A′), NFM (B′), NFL (C′), peripherin (D′), and α-internexin (E′,F′). Panels A, A′ to E, E′ show staining of neurites, while F and F′ show cell body staining.

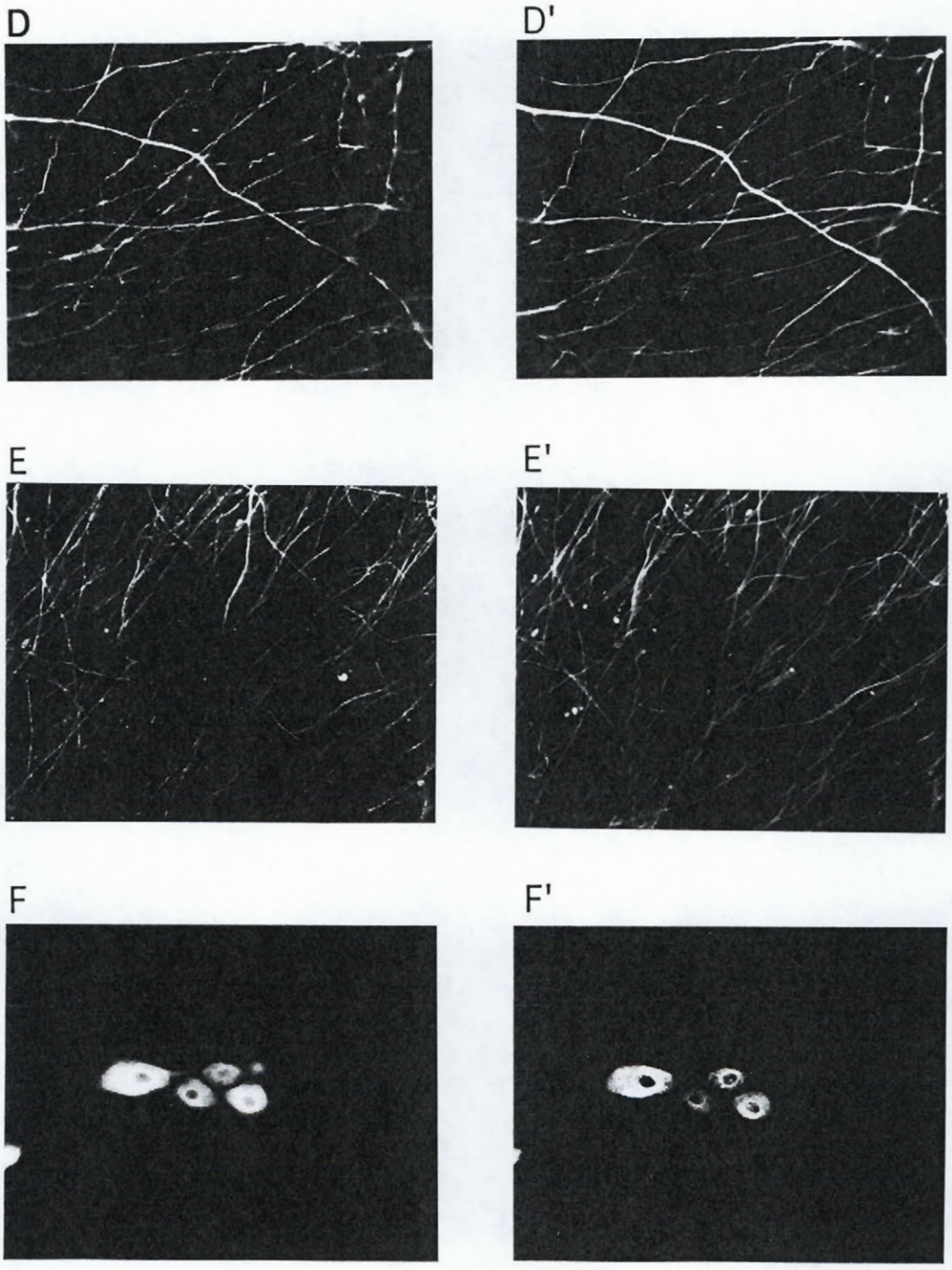

Fig. 3 continued.

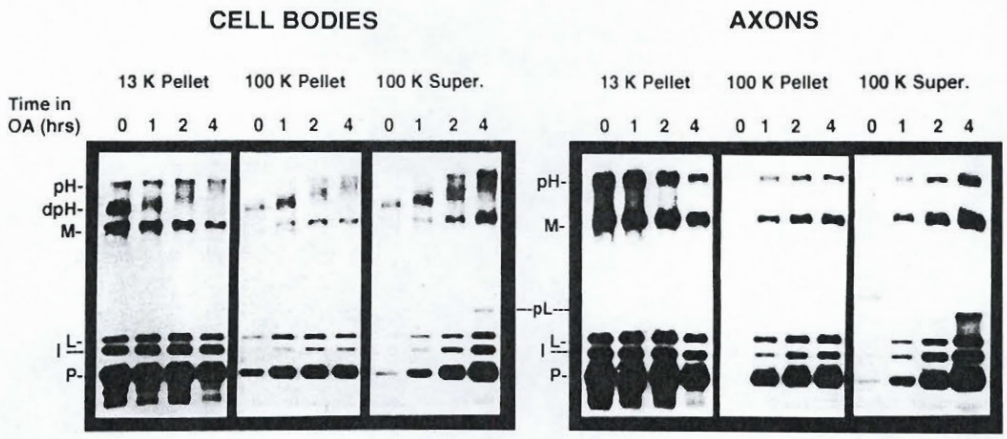

Fig. 4. Western blot analysis of the time-course of neuronal IF fragmentation in OA-treated neurons. Localized DRG cultures were treated with 1 μM OA for 0, 1, 2, or 4 hr. The cell bodies and neurites were separately harvested in CSK buffer and centrifuged at 13,000*g* for 15 min. The pellets (13K pellet) were dissolved in SDS-sample buffer, and the supernatants were centrifuged at 100,000*g* for 5 min, yielding 100,000*g*-insoluble (100K pellet) and -soluble (100K super) fractions. Pellets and supernatants were resolved by SDS-PAGE and analyzed by Western blotting as described in the legend to Figure 2. IF protein bands are designated as described in the legend to Figure 1.

analyzed by immunoprecipitation of the 13K supernatants from cell bodies and neurites with Abs against individual IF proteins. Immunoprecipitates were then analyzed by Western blotting using a cocktail of Abs against all five neuronal IF proteins as probes to determine whether any co-immunoprecipitation was taking place (Fig. 5).

There are three separate controls for the immunoprecipitation data shown in Figure 5. The first (Fig. 5, 50% yield) provides a 50% recovery index as it represents the IF proteins in half of the amount of 13K supernatant used for each immunoprecipitation. The second control (Fig. 5, No Abs) shows that trace amounts of only NFM and α-internexin bound to the anti-mouse IgG1-agarose beads in the absence of an anti-IF antibody. The final control (Fig. 5, 2× dilution) shows successive twofold dilutions of equimolar amounts of individually purified neuronal IF proteins. The strongest signal was obtained with the anti-α-internexin Ab, the weakest with anti-peripherin and anti-NFL, while Abs against NFM and NFH gave more or less equivalent signals.

The anti-NFH immunoprecipitates shown in Figure 5 contained very low amounts of the other neuronal IF proteins, indicating that there was free NFH present in the extracts. However, there were large amounts of NFH in the anti-NFL immunoprecipitates, suggesting the presence of NFH/NFL hetero-oligomers that either contained substoichiometric amounts of NFL or could not be immunoprecipitated by anti-NFH. The next panel (anti-NFM) shows that significant amounts of NFL, α-internexin, and peripherin, as well as small amounts of NFH, co-immunoprecipitated with NFM. The highest level of co-immunoprecipitation was obtained with the anti-NFL Ab, which brought down large amounts of the NF triplet proteins and lesser amounts of α-internexin and peripherin. Anti-α-internexin co-immunoprecipitated NFM and peripherin along with α-internexin. The anti-peripherin immunoprecipitates contained peripherin and α-internexin along with lower amounts of NFM. Only one-fifth of the latter immunoprecipitate was loaded on the gel due to the large amounts of peripherin in our DRG cultures and the particular effectiveness of the anti-peripherin Ab for immunoprecipitation.

To further confirm that co-immunoprecipitation of neuronal IF proteins was not due to the fortuitous association of IF proteins in general, the Western blots were stripped and reprobed with an anti-vimentin Ab. The latter showed that there was virtually no vimentin in any of the immunoprecipitates although an appreciable amount could be seen in the initial Triton-soluble extracts (Fig. 5, bottom panel).

DISCUSSION

In this report, we show that cultured E15 DRG neurons from rat embryos resemble their in vivo counterparts in certain aspects of their developmental expression of neuronal IF proteins. Examination of Figure 2 indicated that α-internexin was the predominant species

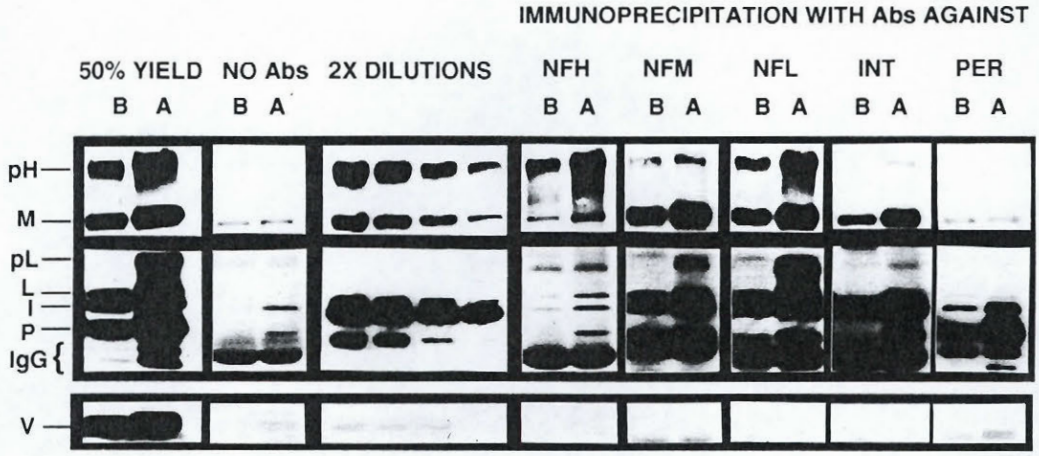

Fig. 5. Western blot analysis of immunoprecipitates from the 13,000*g* Triton-soluble fractions of cell bodies and neurites of OA-treated neurons. Localized DRG cultures were treated with 1 μM OA for 4 hr; the cell bodies and neurites were separately harvested in CSK buffer and centrifuged at 13,000*g* for 15 min. The supernatants were immunoprecipitated as described in Materials and Methods. The panel designations at the top include the following: 50% YIELD, each lane contains one-half of the amount of 13K supernatant fraction used for immunoprecipitation; NO Abs, control with no immunoprecipitating antibody; 2× DILUTIONS, successive twofold dilutions of equimolar amounts of all five neuronal IF proteins. NFH, NFM, NFL, INT, and PER refer to immunoprecipitates obtained with anti-NFH, anti-NFM, anti-NFL, anti-α-internexin, and anti-peripherin, respectively. B and A refer to the cell body and neurite fractions, respectively. Western blots were probed as described in the legend to Figure 2. IF protein bands are designated as described in the legend to Figure 1. Western blots of the various samples and immunoprecipitates were stripped by extraction with 2% SDS, 0.7% β-mercaptoethanol, at 56°C for 30 min and reprobed with an anti-vimentin antibody. The lowest panel shows the vimentin (V) band in these samples. The major band just under peripherin is the IgG heavy chain (IgG).

in freshly dissected DRGs and for the first 2 days after plating, consistent with its early expression in vivo (Fliegner et al., 1994). The early increase in peripherin levels contradicted reports of its tightly coordinated expression with NFL in DRGs (Escurat et al., 1990). On the other hand, the observed co-expression of NFL and NFM concurs with the results of previous studies (Carden et al., 1987), as does the delay in onset of NFH expression (Shaw and Weber, 1982; Pachter and Liem, 1984; Lindenbaum et al., 1988).

As reported previously (Goldstein et al., 1996), E15 DRG neurons fail to differentiate in vitro into two distinct phenotypes expressing either peripherin or NFs. In addition to coexpression of peripherin and NF proteins (Goldstein et al., 1996), we have shown that they retain another embryonic feature, the continued expression of α-internexin. Thus, the reciprocal changes in the levels of NFL and α-internexin expression that occur during development (Fliegner et al., 1990) were not seen. These discrepancies may be due to the inability of cultured neurons to establish contact with appropriate target cells, as attempts to normalize IF protein expression in DRG neurons through the addition of skeletal and heart muscle extracts have met with limited success (Goldstein et al., 1996).

Following the fragmentation pattern of the neuronal IF network in OA-treated cultures provided some insight into the relationship between the different components. In addition to NF proteins, the Triton solubility of peripherin and α-internexin also increased under these conditions. More significantly, the time-course and extent of solubilization were the same for all of these proteins in both the perikaryal and axonal compartments, suggesting that they were interconnected.

OA-induced NF fragmentation is reversible to a point (Sacher et al., 1992) and is likely due to inhibition of protein phosphatase-2A (Cohen et al., 1990; Sacher et al., 1994), an enzyme associated with NFs that has been proposed to play a role in preserving their filamentous structure (Saito et al., 1995). Moreover, NF fragmentation correlates with an increase in the phosphorylation of two protein kinase A sites in the head domain of NFL (Giasson et al., 1996). Head domain phosphorylation may similarly affect α-internexin (Tanaka et al., 1993), but there is a lack of evidence linking peripherin phosphorylation with disassembly (Aletta et al., 1989). Neverthe-

less, it appears that OA shifts the equilibrium between the antagonistic effects of protein phosphatase-2A and protein kinase A (Giasson et al., 1996), apparently magnifying an oligomerization process involved in NF dynamics (Okabe et al., 1993). Thus, IF proteins contained in the oligomeric products of OA-induced disassembly are likely to retain the normal association patterns that exist in situ.

The validity of the co-immunoprecipitation paradigm we used to test for possible associations between the various neuronal IF proteins is based upon several criteria. First, the Western blot in Figure 1 indicated that the individual monoclonal Abs we used did not show any cross-reactivity with other IF proteins. Second, the control in Figure 5 carried out in the absence of primary Abs showed that the fragmented IFs remained soluble throughout the immunoprecipitation protocol. Furthermore, reference to the 50% yield index in Figure 5 indicated that in some cases, co-immunoprecipitated species comprised up to half of the starting material and were likely to be representative of the total fraction. Also noteworthy was the lack of vimentin, a component of satellite cells in DRG cultures, in the immunoprecipitates. All three NF subunits have been shown to co-assemble with vimentin in co-transfected non-neuronal cells (Chin and Liem, 1989, 1990; Monteiro and Cleveland, 1989). The failure of vimentin to co-immunoprecipitate with any of the neuronal IF proteins indicated that the associations we observed did not result from interactions taking place in the Triton extracts following cell lysis.

The co-immunoprecipitation data showed several associations between neuronal IF proteins, but it still is not clear whether the interactions are direct or are due to some protein(s) not detected in these experiments. There is also a problem concerning the apparent lack of reciprocity between co-immunoprecipitates obtained with anti-NFL as compared to those obtained with anti-NFH, anti-α-internexin, and anti-peripherin, respectively. A high level of co-immunoprecipitation of all the neuronal IF proteins was effected by anti-NFL, perhaps reflecting its key role in NF assembly (Ohara et al., 1993). On the other hand, only low amounts of NFL were co-immunoprecipitated by anti-NFH, and none was seen in the anti-α-internexin and anti-peripherin immunoprecipitates. These apparent discrepancies can be explained in several ways. In the case of NFH, it is possible that two forms of this subunit are present in the Triton-soluble fraction, one consisting of free NFH and the other comprising NFH associated with NFL in a form that is refractory to immunoprecipitation by anti-NFH. It is also possible that NFH is associated with hetero-oligomers containing substoichiometric amounts of NFL. However, the latter possibility cannot apply to the anti-α-internexin and anti-peripherin immunoprecipitates because they did not contain NFL. In these two cases it appears that epitope masking may take place in those hetero-oligomers that contain NFL along with α-internexin and/or peripherin. Although the basis for these discrepancies remains to be determined, they do not detract from the validity of our data in demonstrating associations between the various neuronal IF proteins.

The low level of association between NFM and NFH indicated by the co-immunoprecipitation studies suggests that the two subunits may be contained in different subsets of oligomers. This difference may reflect a fundamental aspect of NF organization and could explain the more dynamic nature of NFH in neurons (Takeda et al., 1994).

The present study provides direct evidence for an association between α-internexin, NFM, and peripherin in cultured E15 DRG neurons, indicating that the IF cytoskeleton in these cells is a highly integrated structure. Perhaps the co-assembly of α-internexin and peripherin with NFs produces IFs with a high degree of plasticity required to support neurite outgrowth (Nixon and Shea, 1992). This notion concurs with the presence of high levels of α-internexin in embryonic DRGs (Fliegner et al., 1994) and with the upregulation of peripherin in large NF-containing DRG neurons during axonal regeneration (Oblinger et al., 1989). The elucidation of these and other questions pertaining to the neuronal IF cytoskeleton will no doubt be facilitated by the availability of cultured neurons that co-express all of the major neuronal IF proteins.

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REFERENCES

Aletta JM, Shelanski ML, Greene LA (1989): Phosphorylation of the peripherin 58 kDa neuronal intermediate filament protein: regulation by nerve growth factor and other agents. J Biol Chem 264:4619–4627.

Balin BJ, Lee VM-Y (1991): Individual neurofilament subunits reassembled *in vitro* exhibit unique biochemical morphological and immunological properties. Brain Res 556:196–208.

Balin BJ, Miller ME (1995): Reassembly of the 66 kD neurofilament protein in vitro following isolation and purification from bovine spinal cord. J Neurosci Res 40:79–88.

Carden MJ, Eagles PAM (1986): Chemical cross-linking analyses of ox neurofilaments. Biochem J 234:587–591.

Carden MJ, Trojanowski JQ, Schlaepfer WW, Lee VM-Y (1987): Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. J Neurosci 7:3489–3504.

Chin SSM, Liem RKH (1989): Expression of rat neurofilament proteins NFL and NFM in transfected non-neuronal cells. Eur J Cell Biol 50:475–490.

Chin SSM, Liem RKH (1990): Transfected rat high-molecular-weight neurofilament (NF-H) coassembles with vimentin in a predominantly nonphosphorylated form. J Neurosci 10:3714–3726.

Ching GY, Liem RKH (1993): Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. J Cell Biol 122: 1323–1335.

Chiu FC, Barnes EA, Das K, Haley J, Socolow P, Macaluso FP, Fant J (1989): Characterization of a novel 66 Kd subunit of mammalian neurofilaments. Neuron 2:1435–1445.

Cohen P, Holmes CFB, Tsukitani Y (1990): Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci 15:98–102.

Cohlberg JA, Hajarian H, Tran T, Alipourjeddi P, Noveen A (1995): Neurofilament protein heterotetramers as assembly intermediates. J Biol Chem 270:9334–9339.

Cui C, Stambrook PJ, Parysek LM (1995): Peripherin assembles into homopolymers in SW13 cells. J Cell Sci 108:3279–3284.

Escurat M, Djabali K, Gumpel M, Gros F, Portier M-M (1990): Differential expression of two neuronal intermediate filament proteins peripherin and the low molecular mass neurofilament protein (NF-L) during the development of the rat. J Neurosci 10:764–784.

Fliegner KH, Ching GY, Liem RKH (1990): The predicted amino acid sequence of α-internexin is that of a novel neuronal intermediate filament protein. EMBO J 9:749–755.

Fliegner KH, Kaplan MP, Wood TL, Pintar JE, Liem RKH (1994): Expression of the gene for the neuronal intermediate filament protein α-internexin coincides with the onset of neuronal differentiation in the developing rat nervous system. J Comp Neurol 342:161–173.

Fuchs E, Weber K (1994): Intermediate filaments: structure dynamics function and disease. Annu Rev Biochem 63:345–382.

Geisler N, Weber K (1981): Self-assembly in vitro of the 68000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate sized filaments. J Mol Biol 151:565–571.

Giesler N, Kaufmann E, Fischer S, Plessman U, Weber K (1983): Neurofilament architecture combines structural principles of intermediate filaments with C-terminal extensions increasing in size between triplet proteins. EMBO J 2:1295–1302.

Giasson BI, Cromlish JA, Athlan ES, Mushynski WE (1996): Activation of cyclic AMP-dependent protein kinase in okadaic acid-treated neurons potentiates neurofilament fragmentation and stimulates phosphorylation of Ser-2 in the low-molecular-mass neurofilament subunit. J Neurochem 66:1207–1213.

Goldstein ME, House SB, Gainer H (1991): NF-L and peripherin immunoreactivities define distinct classes of rat sensory ganglion cells. J Neurosci Res 30:92–104.

Golstein ME, Grant P, House SB, Henken DB, Gainer H (1996): Developmental regulation of two distinct neuronal phenotypes in rat dorsal root ganglia. Neuroscience 71:243–258.

Harris J, Ayyub C, Shaw G (1991): A molecular dissection of the carboxy-terminal tails of the major neurofilament subunits NFM and NFH. J Neurosci Res 30:47–62.

Hoffman PN, Lasek RJ (1975): The slow component of axonal transport: identification of major structural polypeptides of the axon and their generality among mammalian neurons. J Cell Biol 66:351–366.

Julien J-P, Mushynski WE (1982): Multiple phosphorylation sites in mammalian neurofilament polypeptides. J Biol Chem 257: 10467–10470.

Kaplan MP, Chin SSM, Fliegner KH, Liem RKH (1990): α-Internexin, a novel neuronal intermediate filament protein precedes the low molecular weight neurofilament protein (NFL) in the developing rat brain. J Neurosci 10:2735–2748.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee MK, Xu Z, Wong PC, Cleveland DW (1993): Neurofilaments are obligate heteropolymers *in vivo*. J Cell Biol 122:1337–1350.

Leonard DGB, Gorham JD, Cole P, Greene LA, Ziff EB (1988): A nerve growth factor-regulated messenger RNA encodes a new intermediate filament protein. J Cell Biol 106:181–193.

Lindenbaum MH, Carbonetto S, Grosveld F, Flavell D, Mushynski WE (1988): Transcriptional and post-transcriptional effects of nerve growth factor on expression of the three neurofilament subunits in PC12 cells. J Biol Chem 263:5662–5667.

Monteiro MJ, Cleveland DW (1989): Expression of NFL and NFM in fibroblasts reveals coassembly of neurofilament and vimentin subunits. J Cell Biol 108:579–593.

Nagakawa T, Chen J, Zhang Z, Kanai Y, Hirokawa N (1995): Two distinct functions of the carboxy terminal tail domain of NFM upon neurofilament assembly: crossbridge formation and longitudinal elongation of filaments. J Cell Biol 129:411–429.

Nixon RA, Shea TB (1992): Dynamics of neuronal intermediate filaments: a developmental perspective. Cell Motil Cytoskeleton 22:81–91.

Oblinger MM, Wong J, Parysek LM (1989): Axotomy-induced changes in the expression of a type III neuronal intermediate filament gene. J Neurosci 9:3766–3775.

Ohara O, Gahara Y, Miyake T, Teraoka H, Kitamura T (1993): Neurofilament deficiency in quail caused by nonsense mutation in neurofilament-L gene. J Cell Biol 121:387–395.

Okabe S, Miyasaka H, Hirokawa N (1993): Dynamics of the neuronal intermediate filaments. J Cell Biol 121:375–386.

Pachter JS, Liem RKH (1984): The differential appearance of neurofilament triplet polypeptides in the developing rat optic nerve. Developmental Biol 103:200–210.

Parysek LM, Chrisholm RL, Ley CA, Goldman RD (1988): A type III intermediate filament gene in expressed in mature neurons. Neuron 1:395–401.

Parysek LM, McReynolds MA, Goldman RD, Ley CA (1991): Some neural intermediate filaments contain both peripherin and the neurofilament proteins. J Neurosci Res 30:80–91.

Portier M-M, de Néchaud B, Gros F (1984): Peripherin a new member of the intermediate filament protein family. Dev Neurosci 6:335–344.

Sacher MG, Athlan ES, Mushynski WE (1992): Okadaic acid induces the rapid and reversible disruption of the neurofilament network in rat dorsal root ganglion neurons. Biochem Biophys Res Commun 186:524–530.

Sacher MG, Athlan ES, Mushynski WE (1994): Increased phosphorylation of the amino-terminal domain of the low molecular weight neurofilament subunit in okadaic acid-treated neurons. J Biol Chem 269:18480–18484.

Saito T, Shima H, Osawa Y, Nagao M, Hemmings BA, Kishimoto T, Hisanaga SI (1995): Neurofilament-associated protein phosphatase 2A: its possible role in preserving neurofilaments in filamentous states. Biochemistry 34:7376–7384.

Shaw G, Weber K (1982): Differential expression of neurofilament triplet proteins in brain development. Nature 298:277–279.

Shaw G, Osborn M, Weber K (1981): An immunofluorescence microscopical study of the neurofilament triplet proteins vimentin

and glial fibrillary acidic protein in the adult rat brain. Eur J Cell Biol 26:68–82.

Shea TB, Sihag RK, Nixon RA (1988): Neurofilament triplet proteins of NB2a/dl neuroblastoma: posttranslational modification and incorporation into the cytoskeletion during differentiation. Dev Brain Res 43:97–109.

Sternberger LA, Sternberger NH (1983): Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. Proc Natl Acad Sci USA 80:6126–6130.

Takeda S, Okabe S, Funakoshi T, Hirokawa N (1994): Differential dynamics of neurofilament-H protein and neurofilament-L protein in neurons. J Cell Biol 127:173–185.

Tanaka J, Ogawara M, Ando S, Shibata M, Yatani R, Kusagawa M, Inagaki M (1993): Phosphorylation of 62 kDa porcine α-internexin, a newly identified intermediate filament protein. Biochem Biophys Res Commun 196:115–123.

Trojanowski JQ, Walkenstein N, Lee VM-Y (1986): Expression of neurofilament subunits in neurons of the central and peripheral nervous system: an immunohistochemical study with monoclonal antibodies. J Neurosci 6:650–660.