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Regulatory and aberrant phosphorylation of neuronal intermediate filament proteins.

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of
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Abstract

The activation of cyclic AMP-dependent protein kinase (PKA) in rat dorsal root ganglion (DRG) cultures, treated concomitantly with low concentrations of okadaic acid that selectively inhibit protein phosphatase-2A, enhanced the disassembly of neuronal intermediate filaments (IFs). The latter disassembly correlated with phosphorylation of the peripherin head domain and a novel, identified PKA site, Ser-2, in the low molecular mass neurofilament (NF) subunit (NFL). On the other hand, insignificant levels of ^{32}P were incorporated into α-internexin under control and experimental conditions that promote disassembly. These findings indicate that phosphorylation of the latter protein is not directly involved in the fragmentation of neuronal IFs. Phosphopeptide mapping of the mid-size NF subunit (NFM) revealed that ^{32}P-labelling of one of its many phosphopeptides is correlated with neuronal IF fragmentation.

The expression and Triton X-100 (Triton) solubility of neuronal IF proteins were determined in the developing rat cerebral cortex. The level of expression of α-internexin was unchanged from embryonic day 15 (E15) to postnatal day 15 (P15), whereas expression of NF subunits increased during this time interval. NFL was largely insoluble in Triton from the time, P5, when there were sufficient amounts for its solubility to be assayed. There was a continual reduction in the Triton solubility of NFM and α-internexin during the E15-P15 developmental period. Similar expression patterns and Triton solubility profiles were obtained for neuronal IF proteins in cultured neurons from E15 cerebral cortex. These results suggest that α-internexin is expressed earlier than NF proteins to provide a more plastic network in the early developing brain.

Correlative studies and direct, *in vivo* activation of stress-activated protein kinases (SAPKs) were used to demonstrate that SAPKs are involved in aberrant phosphorylation of the perikaryal high molecular mass NF subunit (NFH). It was also shown that hyperphosphorylation of perikaryal NFH is a reversible process that does not involve p38 kinases or extracellular signal-regulated kinases (ERKs). The use of defined peptide substrates indicated that SAPKγ preferentially phosphorylates KSPXE motifs in NFH. SAPKγ was shown to be located both in the cell body and neurites of cultured DRG neurons, suggesting that it is likely to be involved in the phosphorylation of cytoplasmic proteins. Collectively, these findings strongly support the notion that activation of SAPKs

causes the aberrant hyperphosphorylation of perikaryal NFH reported in many neurological diseases.

Résumé

L'activation de la protéine kinase dépendante de l'AMP cyclique (PKA) dans des cultures de ganglions dorsaux chez le rat, traitées simultanément avec des concentrations d'acide okadaïque qui inhibitent sélectivement la protéine phosphatase-2A, augmente le désassemblage des filaments intermédaires (FIs) neuronaux. Ce désassemblage coïncide avec la phosphorylation du "domaine de tête" de la périphérine et un nouveau site de PKA, sérine-2, dans la sous-unité des neurofilaments (NFs) de faible poids moléculaire (NFL). Par ailleurs, des niveaux non-significatifs de ^{32}P sont incorporés dans l'α-internexine sous des conditions de contrôle et des conditions expérimentales qui induisent le désassemblage. Ceci indique que la phosphorylation de l'α-internexine n'est pas directement impliquée dans cette fragmentation des IFs. Des profils bidimensionnels de phosphopeptides de la sous-unité des NFs de poids moyens (NFM) ont révélé que le marquage au ^{32}P d'un de ses nombreux phosphopeptides augmente simultanément avec la fragmentation des FIs neuronaux.

L'expression et la solubilité au Triton X-100 (Triton) des protéines des FIs neuronaux ont été déterminées dans le cortex cérébral en développement chez le rat. Le niveau d'expression de l'α-internexine n'a pas changé du jour embryonnaire 15 (E15) au jour postnatal 15 (P15), mais l'expression des sous-unités des NFs a augmenté durant cet intervalle. NFL était en gande partie insoluble au Triton à partir du temps, P5, où sa solubilité pouvait être déterminée. Une réduction continuelle de la solubilité au Triton du NFM et de l'α-internexine durant la période développementale E15-P15 a été observée. Des patrons similaires d'expression et de solubilité au Triton ont été obtenus pour les protéines des FIs neuronaux dans des cultures de neurones de cortex cérébral E15. Ces résultats suggèrent que l'α-internexine est exprimée avant les protéines des NFs pour offrir un réseau filamenteux plus flexible durant le développement du cerveau.

Des études corrélatives et l'induction *in vivo* des protéines kinases activées par le stress (SAPKs) ont été utilisées pour démontrer que les SAPKs sont impliquées dans la phosphorylation de la sous-unité de NFs de haut poids moléculaire (NFH) dans les corps cellulaires. Il a aussi été demontré que l'hyperphosphorylation du NFH dans les corps cellulaires est un processus réversible qui est indépendant des kinases p38 et des kinases

régulées par des signaux extracellulaires (ERKs). L'utilisation de peptides définis comme substrats a démontré que les SAPKs préfèrent phosphoryler les motifs KSPXE du NFH. Il a aussi été démontré que SAPKγ est localisée dans les corps cellulaires et les neurites des neurones de ganglions dorsaux en culture, suggérant que cette kinase est impliquée dans la phosphorylation de protéines cytoplasmiques. Collectivement, ces résultats supportent fortement la notion que l'activation des SAPKs cause l'hyperphosphorylation aberrante du NFH dans les corps cellulaires, rapportée dans plusieurs maladies neurologiques.

Preface

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of paper must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Acknowledgements

First, and foremost, I wish to thank my supervisor Walter Mushynski for his friendship, patience, support, advice and enthusiasm throughout all aspects of this doctoral project.

I would also like to thank Dr. James Cromlish for his help in purifying NFL phosphopeptides by HPLC, and Sylvia Levine for her technical assistance and for creating an enjoyable atmosphere in the laboratory. I am also very grateful for the help and guidance that Eric Athlan, Michael Sacher and Dev Jayaraman provided during the early stages of my doctoral training. I also thank Anne-Claude Gingras and Wendy Bruening for the many fruitful discussions on different aspects of the projects.

I thank Dr. Harrish Pant for the generous gift of NFH peptides, Dr. Michael Karin for his generous gift of MEKK-1 cDNA, Dr. Robert Levine for his help with immunofluorescence microscopy, Dr. Guillermina Almazan for her help in setting up cerebral cortex cultures and Dr. Alex Bell for N-terminal peptide sequencing and helpful suggestions.

The excellent photography by Guy L'Heureux and Carole Verdone-Smith is greatly appreciated.

Original contributions to knowledge

Chapter 2: 1) Revealed that the antagonistic activities of PKA and PP-2A can regulate the assembly state of NFs in cultured DRG neurons.

2) Identified Ser-2 in NFL as a PKA phosphorylation site. To my knowledge, this is the first PKA phosphorylation site identified that does not have a positively charged amino acid residue upstream from the modified Ser or Thr residue.

3) Demonstrated that the phosphorylation of Ser-2 in NFL correlates with NF disassembly in cultured DRG neurons.

4) Demonstrated that phosphoserine-2 and phosphoserine-55 in NFL are substrates for PP-2A, although phosphoserine-2 is a better substrate.

Chapter 3: 1) Demonstrated that peripherin and α-internexin follow the same disassembly profile as NF subunits during simultaneous activation of PKA and inhibition PP-2A in cultured DRG neurons

2) Discovered that phosphorylation of the head domain of peripherin correlates with neuronal IF fragmentation in cultured DRG neurons.

3) Demonstrated that phosphorylation of α-internexin is not involved in the fragmentation of neuronal IFs caused by PKA activation and PP-2A inhibition in cultured DRG neurons.

Chapter 4: 1) Demonstrated that α-internexin is highly soluble in Triton X-100 when it is the predominant neuronal IF subunit expressed in cerebral cortical neurons.

2) Demonstrated that the upregulation of NF subunit levels in the developing cerebral cortex gives rise to a more stable IF network.

3) Provided the first experimental evidence to support the notion that α-internexin is expressed earlier than NF proteins during nervous system development to provide a more plastic IF network.

Chapter 5: 1) Demonstrated a strong correlation between hyperphosphorylation of the carboxyl-terminal domain of NFH and activation of SAPKγ in PC12 cells.

2) Demonstrated that ERKs were not involved in the stress-induced hyperphosphorylation of NFH in PC12 cells.

3) Demonstrated that treatments causing hyperphosphorylation of NFH in PC12 cells have a similar effect on perikaryal NFH in cultured DRG neurons.

4) Demonstrated that SAPKγ preferentially phosphorylates KSPXE motifs in the tail domain of NFH.

Chapter 6: 1) Demonstrated that direct activation of SAPKs by their upstream activator, MEKK-1, causes NFH tail domain hyperphosphorylation *in vivo*.

2) Demonstrated that p38 kinases are not involved in the hyperphosphorylation of perikaryal NFH.

3) Demonstrated that SAPKγ is found in cell bodies and neurites of cultured DRG neurons. This finding implies that SAPKs also have a cytoplasmic role rather than acting solely on transciption factors.

4) Demonstrated that hyperphosphorylation of perikaryal NFH is reversible, thereby suggesting that a phosphatase (s) is likely to be involved in maintaining perikaryal NFH in a hypophosphorylated state.

5) Demonstrated that neuritic NFH is highly phosphorylated in DRG cultures devoid of Schwann cells. This observation indicates that induction of NFH

phosphorylation when it enters axons is not due to cues stemming from Schwann cell-axon contacts.

6) Demonstrated that cdk-5 is inactive in cultured DRG neurons. Since neuritic NFH is highly phosphorylated in these neurons, these findings provide the first evidence indicating that cdk-5 activity is not required for NFH to attain a highly phosphorylated state.

7) Results presented in chapters 6 and 7 strongly suggest that activation of SAPKs is responsible for the aberrant phosphorylation of perikaryal NFH reported in many disease states.

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Abbreviations

α–Int	α-internexin
db cAMP	N^6,2'-O-Dibutyryl cyclic AMP
ALS	Amyotrophic lateral sclerosis
CDK	Cyclin dependent kinase
CI	N-acetyl-Leu-Leu-norleucinal
CII	N-acetyl-Leu-Leu-methioninal
DRG	dorsal root ganglion
EGTA	Ethyleneglycol-bis-(β–aminoethylether)N,N'-tetraacetic acid
ERK	Extracellular regulated kinase
GFAP	Glial fibrillary acidic protein
GSK	Glycogen synthase kinase
GST	Glutathione S-transferase
IDPN	β, β'-iminodipropionitrile
IF	Intermediate filament
KSP	Lysine-serine-proline
MF	Microfilament
MAP	Mitogen-activated protein
MAP	Microtubule-associated protein
MT	Microtubule
NCS	N-chlorosuccinimide
NF	Neurofilament
NFL	Low molecular mass neurofilament protein
NFM	Middle or mid-sized molecular mass neurofilament protein
NFH	High molecular mass neurofilament protein
NGF	Nerve growth factor

OA	Okadaic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PIPES	1, 4-Piperazinediethanesulfonic acid
PKA	Protein kinase A/cAMP-dependent protein kinase
PMSF	Phenylmethylsulfonyl fluoride
PP-1	Protein phosphatase-1
PP-2A	Protein phosphatase-2A
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TNFα	Tumor necrosis factor-α
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	Tris-(hydroxyethyl)-amino methane
Triton	Triton X-100

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Chapter 1

1. Introduction

1.1 The cytoskeleton

The cytoskeleton is the system of fibrillar structures in the cytoplasm of eukaryotic cells. Microfilaments, intermediate filaments and microtubules are the three major types of polymeric filaments which, in association with numerous binding proteins, constitute the cytoskeleton. The term cytoskeleton is undoubtedly appropriate since these filamentous elements form the supporting internal framework of cells. However, inclusion of the word "skeleton" in this term has an unfortune connotation since it implies that the cytosketeton is a static structure. It is actually a highly dynamic matrix involved in intracellular transport, determination of cell shape, cellular motility, cell division and many other essential functions.

The following sections will highlight only the major characteristics of microfilaments and microtubules. The remainder of this introduction will be dedicated to a review of the literature on intermediate filaments, especially aspects pertaining to neuronal cells.

1.1.1 Microtubules

Microtubules (MTs) are 23-24 nm wide filaments consisting of 13 laterally associated, longitudinal rows of globular subunits encircling a hollow-appearing center (Amos and Baker, 1979). The MT subunit, known as tubulin, is a heterodimer containing one α–tubulin and one β-tubulin polypeptide. Tubulin assembles with a specific directionality which endows MTs with a defined polarity, such that the two ends display different assembly kinetics (Kirschner and Mitchison, 1986). In most higher eukaryotic cells, the majority of MTs are nucleated at their so-called minus or slow growing end by MT organizing centers so that only the rapidly growing, plus end is involved in polymerization (Bergen et al., 1980). In neurons, axonal MT arrays run parallel to the axonal axis with the minus end oriented towards the cell body and the plus end towards the axon terminus (Burton and Paige, 1981; Heidemann et al., 1981).

In mammals, six α-tubulin and six β– tubulin genes have been identified (Villasante et al., 1986; Little and Seehaus, 1988). The six α–tubulin genes are more than 94% homologous (Little and Seehaus, 1988). Five of the β-tubulin genes (β2-β6), are

more than 90% homologous, with the greatest variability occurring in the C-termini (Burgoyne et al., 1988; Little and Seehaus, 1988). The expression of tubulin isogenes varies between different tissues. Noteworthy is the finding that the expression of β4 is restricted to the brain although this organ also expresses β2, β3, β5 and β6 (Lewis et al., 1985; 1987; Lewis and Cowan, 1988). The functional differences between the different tubulin isotypes are poorly understood, although biochemical differences have been reported (Luduena et al., 1985; Banerjee and Luduena, 1987; Joshi et al., 1987).

α-tubulin and β-tubulin are both modified post-translationally by two rare, if not tubulin-specific, processes, polyglutamylation and polyglycylation. Polyglutamylation occurs at Glu 445 in α-tubulin (Eddé et al., 1990) and Glu 438 in β-tubulin (Alexander et al., 1991). In the latter modification, the first glutamic acid residue is attached to the γ-carboxyl group, but more distal units may be linked by amide bonds involving either the α– or γ-carboxyl group. Up to 40-50 % of α-tubulin (Edde et al., 1990) and 85 % of class III β-tubulin (Alexander et al., 1991) in the brain are polyglutamylated. Polyglycylation is a potentially bulky modification wherein 3 to 34 glycl units can be convalently attached to the γ-carboxyl group of Glu 445 in α-tubulin or Glu 437 in β-tubulin (Redeker et al., 1994).

α-tubulin is modified by two additional, relatively unusual post-translational modifications. The C-terminal tyrosine is subject to cyclical removal and religation by tubulin carboxypeptidase and ligase enzymes, respectively (Barra et al., 1988). Based on the enzymatic properties of tubulin ligase (Beltramo., 1987) and carboxypeptidase (Kumar and Flavin, 1981), a model has been formulated which proposes that α-tubulin is detyrosinated following its assembly into MTs and re-tyrosinated when tubulin is released during the course of MT disassembly (Barra et al., 1988). The physiological role of tyrosination/detyrosination is poorly understood. The tyrosination state of α-tubulin does not alter the assembly/disassembly properties of tubulin (Paturle et al., 1989). Detyrosinated tubulin is found in stable MTs (Cambray-Deakin, 1987; Kreis, 1987), but the modification is a consequence rather than the cause of stabilization (Khawaja et al., 1988; Webster et al., 1990). Acetylation of the ε amino group of lysine-40 is another post-translational modification that occurs on α-tubulin (L'Hernalt and Rosenbaum, 1985;

LeDizet and Piperno, 1987). Acetylated α-tubulin is frequently co-localized with detyrosinated α-tubulin (Cambray-Deakin, 1987) and MTs containing the former may also turn over more slowly (Webster and Borisy, 1989).

The latest addition to the tubulin gene family is γ-tubulin. It was discovered in the fungus *Asperillus nidulans* as a suppressor of a temperature-sensitive mutation in the β-tubulin gene (Oakley and Oakley, 1989). γ-tubulin is localized at the centrosome and is not detected within the MT lattice itself (Stearns et al., 1991; Zheng et al., 1991). It is involved in the initation of MT assembly and in the anchorage of MTs to MT organizing centers (Stearns et al., 1991; Baas and Joshi, 1992).

MTs are involved in many types of movement, including chromosome segregation and ciliary or flageliar molility. They also serve as tracks for the transport of small vesicles within the cytoplasm. Similarly in neurons, they are involved in anterograde and retrograde axonal transport. Kinesin is the the ATP-dependent, microtubule-based motor involved in anterograde transport (Lasek and Brady, 1985; Vale et al., 1985). Cytoplasmic dynein, also known as MT associated protein (MAP)-1C is the protein motor involved in retrograde transport (Paschal and Vallee, 1987; Paschal et al., 1987).

MAPs are a heterogeneous group of molecules that co-purify with MTs and are particularly abundant in the brain. Tau MAPs comprise a group of six polypeptides, with molecular masses of 55,000 to 62,000 in the adult rat brain, that were first discovered as promoters of tubulin polymerization *in vitro* (Cleveland et al., 1977a, b). Tau was initially reported to be present only in axons (Binder et al., 1985), although it was subsequently shown to also be found in the neuronal cell body and dendrites (Papasazomenos and Binder, 1987). Tau molecules form side-arms that project from the MT surface and appear to cross-link adjacent MTs (Hirokawa et al., 1988). Tranfection studies with tau cDNA demonstrated that tau caused bundling of MTs (Kanai et al., 1989).

Other MAPs expressed in the nervous system include MAPs-1A, -1B, -1C, -2a, -2b, -2c, -3, -4 and -5. MAPs-2a, -2b and -2c are proteins with approximate molecular masses of 280 kD, 280 kD and 70 kD, respectively, which are generated by alternative splicing of a single gene transcript (Garner and Matus, 1988). MAP-2, MAP-1A and MAP-1B are expressed in neurons, although MAPs-1A and -1B are also present in neuroglia (Bloom et al., 1984; 1985). MAP-1A and MAP-1B are found in axons, cell bodies and dendrites (Bloom et al., 1984; 1985) while MAP-2a and MAP-2b are localized to cell bodies and dendrites (Bernhardt and Matus, 1984; Burgoyne and Cumming, 1984; De Camilli et al., 1984). In contrast to the other MAP-2 species, MAP-2c is also present in

axons (Tucker et al., 1988). MAPs-3,-4 and -5 are less abundant than the other MAP species mentioned above. They were identified following the production of antibodies using MT preparations for immunization (Parysek et al., 1984; Huber et al., 1985; Riederer et al., 1986). Most of the MAPs have been shown to promote tubulin polymerization *in vitro* (Sloboda et al., 1976; Cleveland et al., 1977a, b; Kuznetsov et al., 1981; Huber et al., 1986; Riederer et al., 1986).

1.1.2 Microfilaments

Microfilaments (MFs) are 4-6 nm diameter filaments present in all eukaryotic cells (Pollard and Cooper, 1986). These filaments play major roles in many basic cellular functions such as cytokinesis (Schroeder, 1973), endocytosis (Larsen et al., 1979), secretion (Cheek and Burgoyne, 1986; Bernstein and Bamburg, 1989), motility (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996) and cell adhesion (Jones, 1996). In neurons, MFs are especially important for guidance of axonal elongation. They are by far the major cytoskeletal component at the leading edge of the neuronal growth cone since only occasional MTs enter this MF-rich domain (Letourneau, 1983; Bridgeman and Daily, 1989). Experiments with the inhibitor of MF assembly, cytochalasin B, demonstrated the importance of MFs in axonal guidance. Cytochalasin B causes a cessation of filopodial activity, accompanied by the withdrawal of filopodia (Forscher and Smith, 1988). Neurite outgrowth continues in the absence of filopodia, although it becomes undirectional (March and Letourneau, 1984; Bertley and Taroion-Raymond, 1986).

MFs are assembled from monomeric, globular (G)-actin (Korn, 1982). The structure of MFs has been determined based on image reconstruction from crystalline actin sheets (Egelman and DeRosier, 1983) and X-ray crystallographic analysis (Holmes et al., 1990). Actin monomers have a dumbbell structure and they interact to form a helical filament. Decoration of MFs with proteolytic fragments of myosin has demonstrated that they have a defined polarity (Huxley, 1963), which is due to the head-to-tail polymerization of subunits (Korn, 1982).

At lease six different actin genes are expressed in higher vertebrates and the various actins show greater than 94% sequence identity (Vanderckhove and Weber, 1984; Pollard and Cooper, 1986). Two of these genes encode the β and γ non-muscle actins which are expressed in the nervous system. The ratio of β to γ actin isoforms varies from 1:1 to 6:1

depending on the tissue (Otey et al., 1987). The functional basis for the existence of multiple actins isoforms is not clear at present.

Many types of posttranlational actin modification have been reported. Removal of the N-terminal methionine residue and acetylation of the resulting N-terminal aspartate residue (Vanderckhove and Weber, 1984; Solomon and Rubenstein, 1985) as well as methylation of a single histidine residue (Garrels and Gibson, 1976; Saborio and Palmer, 1981) appear to occur in all nonmuscle actins. Phosphorylation and ADP-ribosylation have also been observed (Dunkley and Robinson, 1983; Ohishi and Tsuyama, 1986). The functional significance of most of these post-translational modifications in not known, although ADP-ribosylation increases the monomeric actin concentration in cells and disorganizes the MF network (Reuner et al., 1987).

A large number of proteins bind to G-actin and to MFs. For example, myosin, the thick-filament protein of muscle, is responsible for energy transduction in muscle contraction (Warrick and Spudick, 1987). Numerous accessory proteins have been reported to affect MF dynamics by sequestering actin monomers, or by severing, capping, stabilizing, or bundling MFs. Actin binding proteins are also involved in anchorage of MFs to the plasma membrane (see Pollard and Cooper, 1986; Bamburg and Bernstein, 1991 for reviews).

1.2 Intermediate filaments

Intermediate filaments (IFs) are 10 nm-wide fibers expressed in all higher eukaryotic cells. Their name derives from their intermediate diameter, which falls between those of MFs (4-6 nm) and MTs (23-24 nm). IFs are composed of subunits belonging to the superfamily of IF proteins. IF subunits are expressed in a cell-type specific manner and they have been divided into different types based on sequence homologies in their α-helical rod domain (Table. 1)(Steinert and Roop, 1988; Fuchs and Weber, 1994). Not included in Table 1 is filensin, a novel IF-like protein (Gounari et al., 1993). Filensin is a component of the membrane-skeleton expressed during differentiation of vertebrate lens epithelial cells (Merdes et al., 1991). It can form short 10 nm fibrils *in vitro* and it binds to vimentin (Merdes et al., 1991).

The functions of IFs are poorly understood, although they are believed to regulate cell shape, affect cell adhesion and provide intracellular mechanical strength (Goldman et al., 1996). It has also been proposed that IFs may target specific mRNAs to specific intracellular compartments (Skalli and Goldman, 1996).

Sequence Type	Name	Size (kDa)	Cell-type specific expression
I	Acidic Keratins (~16 proteins)	40-70	Epithelial cells
II	Basic Keratins (~12 proteins)	40-70	Epithelial cells
III	Vimentin	55	Mesenchymal cells
	Desmin	53-54	Muscle cells
	Glial fibrillary acidic protein	50	Glial cells and astrocytes
	Peripherin	53(56-57)	Neurons with peripheral projections
IV	Neurofilament-H	110-115 (200)	Most neurons
	Neurofilament-M	95-100 (150)	Most neurons
	Neurofilament-L	62 (68)	Most neurons
	α-Internexin	55 (66)	Most neurons
V	Lamins (A, B1, B2 and C)	60-70	Nuclear lamina of all higher eukaryotes
VI	Nestin	220	Neuronal and glial progenitors of the CNS and developing skeletal muscle

Table 1. The IF protein family. IF proteins have been divided into different types according to sequence homologies in the α-helical rod domain (Steinert and Roop., 1988). The sizes correspond to the predicted molecular masses of the subunits deduced from their cDNA sequences. The sizes of neuronal IF proteins indicated in parentheses correspond to the apparent molecular masses determined by SDS-PAGE. The information in this table was obtained from Fuchs and Weber, 1994 and Lee and Cleveland, 1996.

1.2.1 IF protein structure

All IF proteins have a highly conserved central α-helical rod domain which is flanked by nonhelical amino-terminal head and carboxy-terminal tail domains (Fig. 1A). These proteins differ from each other biochemically and antigenically mainly due to differences in the sequence and length of their head and tail domains. The rod domains of types I-IV and type VI IF proteins are approximately 310 amino acids in length, while the corresponding domains of type V IF proteins are 42 amino acid longer (Steinert and Roop, 1988; Fuchs and Weber, 1994). Filensin has a shorter rod domain consisting of 281 amino acids (Gounari et al.,1993). The rod domain posseses a heptad repeat sequence pattern of the form $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ (Parry et al., 1977; Steinert and Roop., 1988), important for IF assembly (see section 1.2.2). In most IF proteins, the rod domain is interrupted by three short linkers designated as L1, L12 and L2. L1 and L12 are non- α-helical; L2 is α-helical but does not conform to to a heptad sequence and usually contains a tryptophan residue (Schneidner et al., 1988; Steinert and Roop, 1988). These three interuptions divide the rod domain into four α-helical tracts designated as 1A, 1B, 2A and 2B (Steinert and Roop., 1988; Fuchs and Weber, 1994). The heptad repeats in the mid-sized and heavy molecular mass neurofilament (NF) subunits, NFM and NFH, respectively, are in phase across their L1 linker (Levy et al.,1987; Myers et al., 1987; Napolitano et al., 1987; Lees et al., 1988; Schneidman et al., 1988; Chin and Liem, 1990). These two subunits also lack the proline residue which is found in the L1 linker of many IF proteins (Scheidman et al., 1988). Consequently, the 1A and 1B α-helical tracts of NFH and NFM are continuous.

1.2.2 IF assembly

The first step in IF assembly involves the left-handed intertwining of the rod domains of two subunits to produce a coiled-coil dimer. Coiled-coil dimer formation was originally predicted from X-ray diffraction data (Crick, 1953; Pauling and Corey, 1953). Predictions based on computer analysis of partial IF protein sequences concluded that the most favourable polypeptide interactions would lead to the formation of an in register, parallel dimer (Parry et al., 1977; McLachlan 1978; Steinert et al., 1984). Results of chemical cross-linking at the single cysteine residue of vimentin, desmin, glial fibrillary acidic protein (GFAP) and the low molecular mass NF subunit (NFL) in intact IFs have also been interpreted as supporting the in register, parallel arrangement (Quinlan and

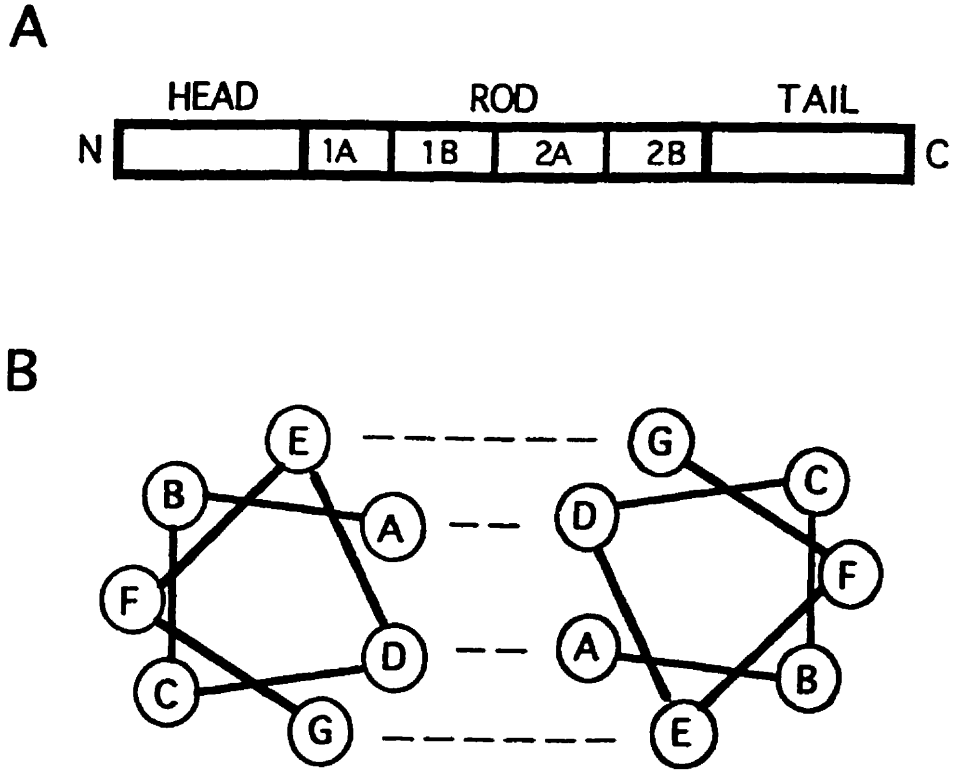

Figure 1. (A) Schematic diagram of IF protein domain organization. Head, rod and tail domains are indicated. The α-helical rod domain is subdivided into 4 segments (1A, 1B, 2A and 2B) due to the presence of 3 linker regions. The amino- and carboxy-termini are labelled N and C, respectively. (B) End-on view of two α-helices interlocking to form a coiled-coil dimer. The sequences have a heptad repeat pattern of residues of the form $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$; a and d are usually apolar, while e and g are frequently of opposite charge and can form a salt bridge.

Franke, 1982; 1983; Carden and Eagles, 1983; Parry et al, 1985). The ability to cross-link homodimeric desmin, vimentin and NFL, dissolved in 3 M guanidine hydrochloride, via their unique cysteine residue also supported this dimer arrangement (Quinlan et al., 1986).

The types of interactions driving coiled-coil dimer formation pertain to the nature of amino acid residues in the heptad repeats. Positions a and d are usually occupied by apolar residues, while b, c, e, f, and g often contain polar or charged residues (Parry et al., 1977; Steinert and Roop, 1988). The a and d residues in two adjacent polypeptides form hydrophobic strips, while e and g residues on adjacent polypeptides and of opposite charge may form salt bridges to further stabilize dimers (Fig. 1B) (Cohen and Parry., 1990).

The second stage of IF protein interaction is at the tetramer level. This progession is supported by evidence such as the isolation of tetrameric cytokeratin complexes in solutions of 4 M urea or 2 M guanidine hydrocloride (Quinlan et al., 1984; Ward et al., 1985) and tetrameric GFAP and NFL in low ionic strength solutions (Rueger et al., 1979; Cohlberg et al., 1987). Furthermore, a small pool of tetrameric vimentin is observable in cells lysed with near-physiological buffer (Soellner et al., 1985). The structure of IF protein tetramers is still controversial. Dimer units have been reported to form parallel (Hisanaga et al., 1990a) or anti-parallel associations (Geisler et al., 1985a; Coulombe and Fuchs, 1990; Steinert and Parry, 1993; Steinert et al., 1993a, b) with each other. Furthermore, two dimers can form tetramers by associating in a close to register arrangement (Coulombe and Fuchs, 1990; Hisanaga et al., 1990a; Steinert and Parry, 1993a, b; Steinert et al., 1993) or in a staggered arrangement (Ip et al., 1985; Coulombe and Fuchs, 1990; Potschka et al., 1990; Steinert and Parry, 1993a, b; Steinert et al., 1993;).

Information concerning higher-order interactions involved in IF structure has been obtained by electron microscopy. Two- to three-nm wide protofilaments are believed to consist of tetramers associated end to end (Aebi et al., 1983; Ip et al., 1985). Two protofilaments appear to associate laterally to form 4.5 nm wide protofibrils (Aebi et al., 1983) and four protofibrils twisted in a right-handed sense appears to form the mature IF (Aebi et al., 1983).

Cytoplasmic IF proteins such as purified cytokeratins, desmin, vimentin, GFAP and NF subunits are capable of forming morphologically normal IFs in the absence of other proteins or co-factors (Steinert et al., 1976; Huiatt et al., 1979; Rueger et al., 1979; Geisler and Weber, 1980; 1981a, b; Zackroff et al., 1982; Liem and Hutchison, 1982; Aebi et al., 1983). Keratin subunits can form homodimers, but they appear to be incapable of forming homo-polymeric filaments (Hatzfeld and Franke, 1985; Hatzfeld and Weber, 1990; Steinert 1990). Rather, keratin polymerization requires a 1:1 ratio of acidic (Type I) to basic (Type

II) keratin subunits (Hatzfeld and Franke, 1985; Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990; Steinert, 1990). Type III IF proteins such as vimentin, GFAP and desmin can form homopolymers *in vitro* and *in vivo* (Huiatt et al., 1979; Rueger et al., 1979; Geisler and Weber, 1980; 1981a; Sarria et al., 1990; Raats et al., 1991). Type III IFs do not coassemble with keratins as evidenced by the finding that two distinct IF networks are formed in cells expressing both vimentin and keratins (Osborn et al., 1980; Tolle et al., 1985).

1.2.3 IF dynamics

IFs are generally presumed to be the most stable component of the mammalian cytoskeleton. One of the major reasons for this notion was the finding that IFs are largely insoluble when cells are lysed in buffers containing non-ionic detergents. However, a variety of biophysical methods have been used to demonstrate that IFs are actually dynamic entities. Fluorescence resonance energy transfer studies with fluorescently labelled NF proteins or desmin revealed that unpolymerized IF proteins exist in a steady-state equilibrium with intact IFs (Angelides et al., 1989). Photobleaching studies with rhodamine-labelled vimentin, NFL or NFH also demonstarted that IFs can accept and exchange subunits along their entire surface (Vikstrom et al.,1992; Okabe et al., 1993, Takeda et al., 1994). Finally, experiments with peptides derived from the helix 1A tract demonstrated their capacity to exchange and cause the disassembly of vimentin IFs both *in vitro* and *in vivo* (Goldman et al., 1996).

1.2.4 Regulation of IF assembly

The finding that IFs rearrange during cell division (Aubin et al., 1980) gave rise to the notion that IF assembly must be regulated. Some of the earliest studies implicating phosphorylation in the regulation of IF assembly were performed *in vitro* using purified type III IF proteins and protein kinases. Phosphorylation of vimentin with protein kinase A/cAMP-dependent kinase (PKA) or protein kinase C caused filament disassembly and blocked assembly (Inagaki et al., 1987; 1988; Geisler et al., 1989). These regulatory phosphorylation events occurred within the head domain of vimentin (Evans 1988; Ando et al., 1989; Geisler et al., 1989). Furthermore, direct injection of PKA into fibroblasts caused the collapse and tight bundling of vimentin IFs around the nucleus (Lamb et al., 1989). Phosphorylation of the head domain of desmin and GFAP was also shown to

regulate the assembly of these filaments *in vitro* (Geisler and Weber, 1988; Inagaki et al., 1988; 1990; Kitamura et al., 1989).

$P34^{cdc2}$ kinase was identified as one of the major protein kinases involved in the phosphorylation of vimentin during mitosis (Chou et al., 1990). Phosphorylation by this kinase is associated with the depolymerization of vimentin IFs to form cytoplasmic aggregates *in vivo* (Chou et al., 1989) and with vimentin disassembly *in vitro* (Chou et al., 1990). Ser 41, Ser 55 and Ser 65 within the head domain of vimentin were identified as the amino acid residues phosphorylated by $p34^{cdc2}$ kinase (Chou et al., 1991). The phosphorylation of GFAP at Ser 8 by $p34^{cdc2}$ kinase has also been implicated in the rearrangement of the glial IF network during the G_2-M phase (Matsuoka et al., 1992).

$P34^{cdc2}$ kinase phosphorylates nuclear lamins *in vitro* at sites comparable to those phosphorylated *in vivo* during mitosis (Peter et al., 1990; Ward and Kirschner, 1990). Modification by the latter kinase also induces lamin network disassembly *in vitro*. Furthermore, double-substituted mutation of lamin A at Ser 22 and Ser 392, two of the identified phosphorylation sites, blocked disassembly of the nuclear lamina during mitosis (Heald and McKeon, 1990).

The importance of protein phosphatases in the regulation of IF dynamics became evident with the availability of specific, cell permeable phosphatase inhibitors. Treatment of cells with okadaic acid or calyculin A, two potent inhibitors of protein phasphatase (PP)-1 and PP-2A (Cohen et al., 1989; Ishihura et al., 1989) caused hyperphosphorylation and fragmentation of vimentin IFs (Yatsumami et al., 1991; Lee et al., 1992; Almazan et al., 1993).

1.3 Neuronal IFs

The earliest IF proteins expressed in and associated with development of the mammalian nervous system are vimentin (Tapscott et al., 1981; Cochard et al., 1984) and nestin (Frederikson and McKay, 1988; Lendahl et al., 1990). However, the expression of both proteins is silenced when neuronal precursor cells become postmitotic. Postmitotic neurons may express some or all of five other IF proteins. Three of these comprise the NF triplet, consisting of NFL, NFM and NFH (Hoffman and Lasek, 1975; Liem et al., 1978; Schlaepfer et al., 1978). α-internexin and peripherin are the other two IF proteins found in neurons (see Table 1 and below).

NFs were first detected as thin fibrous structures in silver stained preparations of many types of neurons. These structures, initially called neurofibrils, were regarded by

many physiologists as an artifact of fixation. Only after the midcentury, when they were observed by electron microscopy as 10 nm diameter filaments, were NFs acknowledged to be real organelles (Schmitt, 1968).

NFM is the first NF subunit to be expressed in many types of neurons during development. (Bennett and Dilullo, 1985a; Szaro et al., 1989; Schlaepfer and Bruce, 1990). The expression of NFL reportedly occurs simultaneously or soon after NFM (Shaw and Weber, 1982; Willard and Simon 1983; Pachter and Liem, 1984; Carden et al., 1987; Schlaepfer and Bruce, 1990; Athlan et al., 1997). On the other hand, NFH expression has been consistently reported to occur later than the other neuronal IF proteins during development (Shaw and Weber, 1982; Willard and Simon 1983; Pachter and Liem, 1984; Carden et al., 1987; Athlan et al., 1997). In large myelinated axons, NFs acccumulate to become the most abundant fibrillar element, outnumbering MTs by as much as 10 to 1 (Hoffman et al., 1984).

α-internexin was originally purified as a NF binding protein (Pachter and Liem, 1985). It was later identified as a true neuronal IF protein (Chiu et al., 1989; Kaplan et al., 1990; Fliegner et al., 1990). Its expression precedes that of the other neuronal IF proteins and it is down-regulated in the aging nervous system (Chiu et al., 1989; Kaplan et al., 1990; Fliegner et al., 1990; 1994). α-internexin appears to be found in most, if not all, neurons.

Peripherin was intially identified as a 56 kD Triton-insoluble protein present in PC12 cells and murine neuroblastoma cells (Portier et al., 1984a). Since the protein was detected in sympathetic and sensory neurons, but not in nonneuronal cells or in several brain regions, it was named peripherin (Portier et al., 1984a,b). Based on its immunoreactivty with IF-specific antibody, filamentous staining pattern and single centrally located tryptophan residue, peripherin was proposed to be a novel IF protein (Portier et al., 1984b). Peripherin was confirmed to be an IF protein when its cDNA was cloned by two different groups. Parysek and co-workers purified peripherin as a 57-kDa Triton-insoluble protein that could form 10 nm filaments *in vitro* (Parysek and Goldman, 1987) and they eventually cloned its cDNA (Parysek et al., 1988). At about the same time, another group cloned the cDNA of a novel IF protein while screening for messenger RNAs that were induced when PC12 cells were challenged with nerve growth factor (NGF) (Leonard et al., 1988). This novel IF protein was subsequently shown to be peripherin (Aletta et al., 1988). Peripherin is expressed in neurons of the peripheral nervous system as well as in CNS neurons which extend axons to the periphery (Leonard et al., 1988; Parysek and Goldman, 1988; Escurat et al., 1990).

1.3.1 Distinctive features of NF subunits

The main distinguishing feature of NF subunits compared to other IF proteins is their tail domain. For NFL, this region is highly acidic, with many glutamic acid residues (Geisler et al., 1985b; Julien et al., 1986; 1987; Chin and Liem, 1989). NFM and NFH have unusually long tail domains. The NFM tail domain contains many glutamic acid-rich and glutamic acid/lysine-rich segments (Levy et al., 1987; Napolitano et al., 1987; Myers et al., 1987). Lysine-serine-proline (KSP) repeats are a feature of both NFM and NFH tail domains, which contain 4 to 13 and 42 to 55 of these repeats, respectively (Levy et al., 1987; Myers et al., 1987; Napolitano et al., 1987; Lees et al., 1988; Julien et al., 1988; Schneidman et al., 1988; Chin and Liem, 1990; Soppet et al., 1992) .

NFM and NFH purified from axons have apparent molecular masses of approximately 150 kDa and 200 kDa, respectively, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), (Hoffman and Lasek, 1975; Liem et al., 1978; Schlaepfer and Freeman, 1978) as compared to 95-100 kDa and 110-115 calculated from from their deduced amino acid sequences. The anomalous electrophoretic mobility is partly due to the high content of phosphate moieties in the tail domains of both proteins (see section 1.3.4). The high content of carboxylic amino acid residues in the tail domain also contributes to the aberrant mobility of these proteins on SDS-PAGE (Georges and Mushynski, 1987).

1.3.2 NF ultrastructure

NFs are composed of a longitudinal core structure with many short protrusions (Hirokawa, 1982; Hisanaga and Hirokawa, 1988). Many studies support the notion that these side-arms are the tail domains of NFM and NFH extending laterally from the filament core. Homopolymeric NFL filaments are smooth-walled, without the protrusions characteristic of native NFs. Reassembly of NFL in the presence of NFH or NFM restores the side-arms (Hisanaga and Hirokawa 1988; 1990; Gotow et al., 1992). In addition, the side arms are decorated only by antibodies directed against the tail domains of NFH and NFM (Hirokawa et al., 1984; Mulligan et al., 1991). However, it is unlikely that NFH and NFM are located solely at the periphery of NFs. NFM and NFH preferentially pair with NFL to form dimers (Carpenter and Ip., 1996; Leung and Liem, 1996). In 2 M urea, they can form stable tetramers only in the presence of stoichiometric quantities of NFL (Cohlberg et al., 1996). Furthermore, the ratio of NFM plus NFH to NFL *in vivo* has

been reported to be within the range of 0.5: 1 to 1.25:1 (Shecket and Lasek, 1980; Moon et al., 1981; Chiu and Norton, 1982; Scott et al., 1985). Taken together, these results support the idea that all three NF subunits are integral components of NFs.

Axonal NFs run parallel to each other and side-arms appear to form cross-bridges between adjacent NFs (Hirokawa, 1982; Hirokawa et al., 1984; Nakagawa et al., 1995). Side-arms also appear to contact MTs and membranous organelles (Hirokawa 1982; Nakagawa et al., 1995).

1.3.3 Neuronal IF assembly

Purified NF triplet proteins can reassemble into 10 nm filaments when dialyzed from denaturing solutions into solutions of physiological ionic strength (Liem and Hutchison, 1982; Zachroff et al., 1982; Carden and Eagles, 1983). NFL alone can also reassemble into smooth-walled 10 nm filaments *in vitro* (Geisler and Weber, 1981b; Liem and Hutchison, 1982; Zachroff et al., 1982; Hisanaga and Hirokawa, 1988; 1990). Combinations of NFL and NFM or NFL and NFH also reassemble into 10 nm filaments (Geisler and Weber, 1981b; Liem and Hutchison, 1982; Hisanaga and Hirokawa, 1988; Balin and Lee, 1991). NFM and NFH alone or in combination are incapable of forming complete filaments (Geisler and Weber, 1981b; Liem and Hutchison, 1982; Zachroff et al., 1982). However, NFM self-assembles into short, irregular 10 nm filaments under optimal conditions (Balin and Lee, 1991; Gardner et al., 1984).

Although NFL can form homopolymeric filaments *in vitro*, NFs are obligate heteropolymers *in vivo*. DNA transfection studies in a cell line deficient in endogenous cytoplasmic IFs (SW 13$^-$) demonstrated that neither of the NF subunits alone could assemble *in vivo* (Ching and Liem, 1993; Lee et al., 1993). Assembly in these cells required NFL and a substoichiometric amount of either NFM or NFH. The heteropolymeric requirement for NF subunit assembly was also studied in oligodendrocytes of transgenic mice. In these cells, which are normally devoid of IFs, extended filamentous arrays were observed only when both NFL and NFM transgenes were co-expressed. IFs were not observed in oligodendrocytes expressing only one of these transgenes (Lee et al., 1993). Furthermore, because a hybrid NF subunit composed of the NFH tail fused to the NFL head and rod assembled with NFM, it was concluded that steric hindrance involving the unusually long tail domains of NFH and NFM was not responsible for their inability to form homoplymers (Lee et al., 1993). Co-expression of the NFH/NFL hybrid protein with NFL produced nonfilamentous aggregates, indicating that polypeptide sequences in the head and/or rod domain of NFH are required for

heteropolymeric assembly. The preference for heterologous assembly was also observed in Sf9 insect cells (Nakagawa et al., 1995). Although high expression levels of NFL can lead to assembly in these cells, most of the NFL was found in nonfilamentous aggregates. Coexpression of NFM and NFL eliminated nonfilamentuous aggregates and produced long bundles of filaments.

The stage of NF assembly at which heterologous subunit interactions are essential is still unclear. Analysis of assembly intermediates in 2M urea, showed that NFL can associate with a stochiometric amount of NFM or NFH to from stable tetramers. Under the same conditions, NFH and NFM are monomers or perhaps, monomers in equilibrium with dimers (Cohlberg et al., 1996). Studies using the yeast two hybrid system demonstrated that NFL can interact with itself, with NFM or with NFH (Carpenter and Ip, 1996; Leung and Liem, 1996). No homologous or heterologous NFM and NFH interactions were detected in this system. These studies suggest that NFL/NFL, NFL/NFM and NFL/NFH dimers are more stable than dimers containing only NFM or NFH.

Unlike the other type IV IF subunits, α-internexin can assemble into an extended homopolymeric network both *in vivo* and *in vitro* (Chiu et al., 1989; Kaplan et al., 1990; Ching and Liem, 1993; Balin and Miller, 1995). α-internexin can also coassemble with either of the NF subunits (Ching and Liem, 1993; Balin and Miller, 1995).

Peripherin, like other type III IF proteins (see section 1.2.2), can form homopolymeric 10 nm filaments *in vitro* and *in vivo* (Parysek and Goldman, 1987; Parysek et al., 1988; Cui et al., 1995). Double-label immunofluorescence and immunoelectron microscopy studies in mammalian neurons and neuron-like cultured cells that express NF subunits demonstrated that NF subunits and peripherin colocalized in some IFs (Parysek et al., 1991). Also noteworthy is the observed co-immunoprecipitation of α-internexin, peripherin and NF subunits from extracts of okadaic acid-treated sensory neurons, suggesting that these proteins form a highly integrated network in these cells (Athlan et al., 1997).

1.3.4 Neuronal IF phosphorylation

NFM and NFH are highly phosphorylated *in vivo* , each protein containing from 6 to 16 and 13 to 57 moles of phosphate per mole of polypeptide, respectively (Jones and Williams, 1982; Julien and Mushynski, 1982; the reported values have been corrected to reflect more recent determinations of the molecular masses of the subunits). The

development of monoclonal antibodies that could distinguish between phosphorylated and unphosphorylated epitopes in the tail domains of NFM and NFH led to the discovery that both proteins are normally more highly phosphorylated in the axon than in the perikaryon (Sternberger and Sternberger, 1983; Lee et al., 1987).

NFH is synthesized and maintained in the cell body as a hypophosphorylated isoform (s) with an apparent molecular mass of 160 kDa on SDS-PAGE. When NFH is transported from the cell body to the axon, it is phosphorylated within the proximal region of the axon and its apparent molecular mass on SDS-PAGE increases to 200 kDa (Glicksman et al., 1987; Nixon et al., 1989; Oblinger et al., 1989). The majority of NFH phosphorylation occurs at KSP repeats in the tail domain (Julien and Mushynski, 1983; Lee et al., 1988; Elhanany et al., 1994). The gel electrophoretic mobility of axonal NFH can be increased to that of perikaryal NFH by dephosphorylation of the tail domain *in vitro* (Julien and Mushynski., 1982; Carden et al., 1985). This shift can be reversed by phosphorylation at KSP repeats (Hisanaga et al., 1991, 1993b; Miyasaka et al., 1993).

Several neuronal proline-directed protein kinases have been shown to phosphorylate the KSP repeats in NFH. Extracellular signal-regulated kinases (ERKs) (Roder and Ingram, 1991; Roder et al., 1995) and tau protein kinase I (Hisanaga., 1993b), later identified as glycogen synthase kinase-3β (GSK-3β) (Ishiguro et al., 1993), were able to phosphorylate NFH *in vitro*, but not to levels sufficient to reduce its mobility on SDS-PAGE to that of axonal NFH. Co-transfection studies with NFH and GSK-3α or GSK-3β caused only a partial shift in NFH mobility on SDS-PAGE (Guidato et al., 1996). *In vivo* and *in vitro* phosphorylation by cyclin-dependent kinase-5 (cdk-5), also known as tau protein kinase II (Kobayaski et al., 1993), has being shown to cause a reduction in the mobility of NFH on SDS-PAGE to levels seen for axonal NFH (Hisanaga 1993b; Miyasaka et al., 1993; Guidato et al., 1996; Sun et al., 1996). However, cdk5 has been reported to add only 3-5 (Miyasaka et al., 1993) or 10 (Hisanaga et al et al.,1993b) moles of phosphate per mole of NFH, presumably at KS/TPXK motifs, where X is not an acidic residue (Beaudette et al., 1993). $P34^{cdc2}$ kinase can also phosphorylate NFH *in vitro* to the extent that it reduces its mobility on SDS-PAGE (Hisanaga et al., 1991; Guan et al., 1992;). However, since the latter enzyme is not expressed in post-mitotic neurons it cannot be involved in the phosphorylation of NFH *in vivo*.

Murine NFM is synthesized in the cell body as a polypeptide with an apparent molecular mass on SDS-PAGE of 139 kDa. It appears in the axon as many isoforms with apparent molecular masses of 140-145 kDa (Nixon et al., 1989). Chicken, NFM exhibits an even greater change in mobility between both cellular compartments: 130 kDa in the cell

body as compared to 160 kDa in axons (Bennett and DiLullo., 1985b).This reduction in mobility has been attributed to phosphorylation since it can be reversed by *in vitro* dephosphorylation (Carden et al., 1985; Georges et al., 1986; Nixon et al., 1989). The major NFM phosphorylation sites are localized in the tail domain (Julien and Mushynski, 1983; Geisler et al., 1987; Lee et al., 1988). At least seven serine residues in the tail domain of rat NFM are phosphorylated *in vivo*. Four of these sites (Ser 502, Ser 506, Ser 603 and Ser 606) are within KSP motifs, while Ser 666 and Ser 766 are within ESP and VSP motifs, respectively (Xu et al., 1992; Betts et al., 1997). It is not certain which kinases phosphorylate these sites *in vivo*, although many proline-directed kinases have been shown to phosphorylate NFM *in vitro*. GSK-3α phosphorylates Ser 502, Ser 506, Ser 603 and Ser 666 *in vitro* (Yang et al., 1995). GSK-3β, cdk-5 and $p34^{cdc2}$ kinase also phosphorylate NFM *in vitro*, but only ERKs have been shown to phosphorylate it to an extent that reduces its mobility on SDS-PAGE (Hisanaga et al., 1991; 1993b; Roder and Ingram, 1991; Guan et al., 1992; Roder et al., 1995). Ser 538 is the only identified phosphorylation site in the tail domain of rat NFM which is not in a Ser-Pro motif (Xu et al.,1992). The homologous site in chicken NFM is phosphorylated by casein kinase I (Hollander et al., 1996).

Ser 502, Ser 528 and Ser 536 have been identified as *in vivo* phosphorylation sites in the tail domain of chicken NFM (Hollander et al., 1996). These sites are phosphorylated by a casein kinase-1-like enzyme that co-purifies with NFs (Link et al., 1993; Hollander et al., 1996). These three serine residues in chicken NFM are surrounded be acidic amino acid residues and they are not located within Ser-Pro motifs (Hollander et al., 1996).

The head domain of NFM is also phosphorylated *in vivo*, but to a much lower extent than the tail domain (Sihag and Nixon, 1990). The head domain phosphopeptides derived from NFM phosphorylated *in vivo* are also generated when NFM is phosphorylated by PKA or protein kinase C *in vitro*, suggesting that the latter two kinases may phosphorylate NFM *in vivo* (Sihag and Nixon, 1990). It was recently determined that Ser 23 and Ser 41 in NFM are prefertentially phosphorylated by protein kinase A *in vitro*, although Ser 28, Ser 32, and Ser 48 are also phosphorylated to a lesser extent (Sihag et al., 1995). Protein kinase C modifies six serine residues within the head domain of NFM, three of which are the same ones phosphorylated by PKA (Sihag et al., 1995).

NFL contains between 2.2 and 3.1 moles of phosphate per mole of polypeptide (Julien and Mushynski, 1982; Xu et al., 1990). The major *in vivo* phosphorylation site(s) in NFL is located within an 11 kDa polypeptide which is most likely derived from the tail domain and corresponds to amino acid residues 443-541 (Sihag and Nixon., 1989). The

major phosphorylation site, modified at approximately 73%, is Ser 473 (Xu et al., 1990). The enzyme that phosphorylates this site is not known for certain. However, since Ser 473 and the 11 kDa NFL polypeptide are phosphorylated by the NF-associated kinase (s) (Sihag and Nixon., 1989; Xu et al., 1990), which is mainly a CKI-like activity (Link et al., 1993; Hollander et al., 1996). NFL is reportedly phosphorylated at two other, less intensely labelled sites designated as L-2 and L-3 (Sihag and Nixon., 1989). L-2 was identified as Ser-55 in the head domain (Sihag and Nixon, 1991). This serine residue is a substrate for PKA and its phosphorylation displays rapid turnover immediately following NFL synthesis in neurons (Sihag and Nixon, 1991). These characteristics of Ser-55 phosphorylation raise the possibility that it may be involved in regulation of assembly prior to NF transport into neurites (Sihag and Nixon., 1991).

Peripherin is phosphorylated in PC12 cells and this process is enhanced upon addition of nerve growth factor (Aletta et al., 1989). Peripherin is also phosphorylated in a neuroblastoma cell line and in cultured sympathetic neurons. Phosphorylation occurs almost exclusively within the amino-terminal half of the protein in the latter two cell types (Huc et al., 1989)

1.3.5 Regulation of neuronal IF assembly

The *in vitro* phosphorylation of NFL by either PKA or protein kinase C can block its polymerization or cause disassembly of homopolymeric NFL (Gonda et al., 1990; Hisanaga et al., 1990b; Nakamura et al., 1990). Incorporation of 1 to 2 moles of phosphate per mole of NFL polypetide was sufficient to block assembly (Hisanaga et al., 1990b; Nakamura et al., 1990) at the octomeric intermediate stage (Hisanaga et al., 1990b). The sites preferentially phosphorylated by either PKA or protein kinase C are localized within the head domain of NFL (Gonda et al., 1990; Hisanaga et al., 1994). The inhibition of NFL assembly due to PKA phosphorylation can be reversed by *in vitro* dephosphorylation with the catalytic subunit of PP-2A (Saito et al., 1995). It is interesting to note in this regard that the catalytic subunit of PKA and a PP-2A-like enyzme are associated with native NFs (Dosemeci and Pant, 1992; Saito et al., 1995).

There is also *in vivo* evidence to support the proposal that NFL phosphorylation plays a role in regulating NF assembly. Treatment of cultured dorsal root ganglion neurons with the protein phosphatase inhibitor, okadaic acid, caused a reversible disruption of the NF network (Sacher et al., 1992). This disruption was associated with phosphorylation of serine residues in the head domain of NFL (Sacher et al., 1994).

NFs composed of all three subunits are more resistant to phosphorylation-induced disassembly than are NFL homopolymers (Hisanaga et al., 1994). Only limited disassembly of NFs was observed even after extensive phosphorylation with PKA. Phosphorylation of NFL or NFM with PKA can inhibit NFL/NFM coassembly (Streifel et al., 1996). This phosphorylation had no effect on NFL/NFM heterooligomer formation, indicating that the modification was affecting later stages of assembly. Phosphorylation of purified NFM or NFH with PKA prevented aggregate formation *in vitro* (Streifel et al., 1996). The latter observation suggests that this reversible process may be important for regulating the first step of assembly following synthesis of the proteins. PKA can also phosphorylate and regulate the assembly of α-internexin *in vitro* (Tanaka et al., 1993).

The amino-terminal head regions of NFL, NFM and NFH isolated from rat spinal cord are posttranslationally modified by addition of O-linked N-acetylglucosamine moieties (Dong et al., 1993; 1996). However, it is still not known whether these modifications have any effect on the assembly of NFs.

1.3.6 Axonal transport of NFs

NFs are synthesized in the perikaryon and move down the axon in the slow axonal transport compartment (Hoffman and Lasek, 1975) until they reach the nerve terminal, where they are degraded (Roots, 1983). The axonal transport of NFs has been reported to occur at a rate ranging from .25 to 3 mm/day (Hoffman and Lasek, 1975; Black and Lasek, 1980; Komiya, 1980; Hoffman et al., 1983). NFs are transported more rapidly in immature than in mature axons (Komiya, 1980; Hoffman et al., 1983; Willard and Simon, 1983) and the velocity is further reduced in aging animals (McQuarrie et al., 1989). The slowing of axonal transport during development correlates with an increase in the expression of NFH (Willard and Simon, 1983). The rate of NF axonal transport is also dependent on the metabolic state of the animal. Most notably, the rate of transport decreases in diabetic animals (Medori et al., 1985; 1988).

The mechanism by which NFs are transported from the cell body to the axon, and down the axon, remains unsettled. Experiments with the neurotoxin, β,β'-iminodipropionitrile (IDPN), suggests that association with MTs is involved. IDPN causes NFs and MTs to segregate; NFs become localized almost exclusively at the perimeter of the axon while the MTs are located at the center (Griffin et al., 1978; Papasozomenos et al., 1981). At appropriate doses, IDPN blocks the movement of NFs but has only a modest

effect on tubulin transport (Griffin et al., 1978). This treatment causes NFs to accumulate in large masses at the proximal end of the axon near the cell body (Chou and Hartman, 1965). Within this context, the finding that NFH interacts with MTs only when its tail domain is hypophosphorylated (Hisanaga et al., 1991; 1993a, b; Miyasaka et al., 1993), suggests that NFH tail domain phosphorylation may be important for regulating NF transport from the cell body to the axon, and perhaps along the axon.

NF axonal transport studies remain controversial. Initial studies were interpreted to suggest that all three NF subunits are transported together in a single wave (Hoffman and Lasek, 1975; Black and Lasek, 1980). More recent reexamination of NF transport revealed the existence of two pools of axonal NFs, one moving at the traditionally slow rate and the other being essentially stationary (Nixon and Logvinenko, 1986). Furthermore, highly phosphorylated NFH was demonstrated to be preferentially associated with the stationary pool, suggesting that tail domain phosphorylation may regulate its distribution between stationary and mobile phases (Lewis and Nixon, 1988). However, the proportion of axonal NFs contained in the stationary phase remains controversial and unresolved. Nixon's group estimated that approximately one-third of axonal NFs are in the stationary phase (Nixon and Logvinenko, 1986), while Lasek and co-workers argued that this phase is negligible in size (< 0.1%) (Lasek et al., 1992). It has also been observed that NF subunits can move along axons as fast as 72 to 144 mm/day (Lasek et al., 1993). This finding has led to the proposal that NFs may be transported in alternative periods of short, fast strides and relatively long pauses (Lasek et al., 1993).

The question of whether NFs are transported as filaments or as oligomers is also a controversial issue. In the classic paradigm, NFs are thought to be transported as polymers that slide past each other as they move down the axon (Lasek, 1986; Lasek et al., 1992). On the other hand, photobleaching studies with fluorescently tagged NFL or NFH revealed that bleached zones can recover without translocation (Okabe et al., 1993; Takeda et al., 1994). These studies suggest that NF subunits can exchange between filaments and a small oligomer pool. Furthermore, studies of NFM transport in mice lacking axonal NFs demonstrated that NFM could be transported down axons in an unpolymerized form (Terada et al., 1996).

1.3.7 NFs are an important determinant of axonal caliber

Many correlative studies have implicated NFs as an important determinant of axonal caliber. These studies showed a linear relationship between axonal cross-sectional area and

NF number (Friede and Samorajski, 1970; Weiss and Mayr, 1971; Hoffman et al., 1984; 1985a, b; 1987). The importance of NFs in specifying axonal caliber has recently been proven unequivocally in two different animal species. The expression of a NFH-beta-galactosidase fusion protein in transgenic mice caused a total inhibition of NF axonal transport coupled with severe inhibition of the radial growth of large myelinated axons (Eyer and Peterson, 1994). A reduction in axonal caliber was also observed in large myelinated axons from mice deficient in axonal NFs due to the targetted disruption of the gene encoding NFL (Zhu et al., 1997). The Japanese quail quiver (quv) mutant is deficient in axonal NFs due to a lack of NFL stemming from a nonsense mutation in the NF-L gene (Yamasaki et al., 1991; Ohara et al., 1993). The axons in these quail have a smaller diameter and there is a consequent reduction in axonal conduction velocity (Yamasaki et al., 1991; Sakaguchi et al., 1993). Furthermore, these birds exhibit quivering and generalized ataxia.

The mechanism through which NFs influence axonal redial growth remains unsettled. Overexpression of NFL leads to an increase in axonal NF density and a slight reduction in axonal caliber (Monteiro et al., 1990). Overexpression of NFL together with either NFM or NFH is required to produce an increase in axonal caliber (Xu et al., 1996), suggesting that interactions involving the long tail domains of NFH and NFM have an influence on axonal diameter. The overexpression of NFH or NFM individually appears to change the ratio of NF subunits and cause reductions in NF transport and axonal caliber (Marszalek et al., 1995; Wong et al., 1995; Xu et al., 1996).

1.3.8 Possible role of neuronal IF proteins in axonal elongation

Several reports suggest the possible involvement of neuronal IF proteins in axonal elongation. Noteworthy is the upregulation of two atypical neuronal IF proteins, referred to as plasticin and gefiltin, following axotomy of the goldfish optic nerve which has led to speculation that these proteins may be involved in plasticity and regeneration (Glasgow et al., 1992; 1994; Fuchs et al., 1994). More direct evidence comes from the microinjection of anti-NFM antibodies into single blastomeres of two cell-stage *Xenopus laevis* embyros. This procedure caused neuronal IF accumulation, retardation of peripheral nerve growth and reduction in neurite outgrowth of cultured embyonic spinal cord neurons (Szaro et al., 1991; Lin and Szaro, 1995).

Two recent studies also support the notion that NFs are involved in regeneration. Analysis of NF expression in regenerating spinal axons of the primative fish, sea lamprey, suggests that NFs may be involved in the forward movement of some regrowing axons

following spinal transection (Jacobs et al., 1997). In addition, there is a delayed regeneration of myelinated axons following axotomy of peripheral nerves in mice deficient in NFs due to the targetted disruption of the gene encoding NFL (Zhu et al., 1997).

1.3.9 NFs in disease

Abnormal accumulations of NFs have been associated with several neurological diseases. In Pick's disease and Parkinson's disease certain neurons contain characteristic cytoplasmic inclusions which are known, respectively, as Pick's bodies and Lewy bodies. Pick's bodies are round structures found in the central cortex and basal ganglia and are composed of granular material together with a heterogeneous collection of filaments with a diameter of 10 to 20 nm (Rewcastle and Ball, 1968). Immunocytochemical studies have revealed the presence of NF antigens in Pick's bodies (Probst et al., 1983; Munoz-Garcia and Ludwin, 1984). Lewy bodies are spheroidal inclusions composed of filaments similar in diameter to NFs (Duffy and Tennyson, 1965) and are recognized by antibodies directed against NFs (Goldman et al., 1983; Forno et al., 1986; Pollanen et al., 1994).

Immunocytochemical studies had suggested that NFs were a component of neurofibrillary tangles in Alzheimer's disease (Sternberger et al., 1985; Cork et al., 1986). However, some of the NF antibodies that were used to label these neurofibrillary tangles were shown to cross-react with tau proteins (Kukina et al., 1987; Reding-Ksiezak et al., 1987), which are also found in these structures (Kondo et al., 1988; Wischik et al., 1988). Whether NFs are present in Alzheimer's neurofibrillary tangles is still a controversial issue, although at least one study still supports the notion that they are (Zhang et al., 1989).

The role of NF inclusions in the diseases mentioned above remains unsettled, although an increasing body of evidence supports the notion that NFs are directly involved in the etiology of amyotrophic lateral sclerosis (ALS). ALS is a neurodegenerative disease that targets large, NF-rich motor neurons predominantly and large sensory neurons to a lesser degree (Tsukagoshi et al., 1979; Kawamura et al., 1981). Large proximal axonal swellings filled with NFs are a hallmark of the disease (Carpenter, 1968; Hirano et al., 1984; Manetto et al., 1988; Munoz et al.,1988; Sobue et al., 1990) and abnormal hyperphosphorylation of perikaryal NFH has also been reported in these neurons (Manetto et al., 1988; Sobue et al., 1990). Several transgenic mouse studies indicate that perturbation of NF homeostasis brought about by mutation, or overexpression of individual NF subunits, can cause pathological neurofilamentous accumulations in the perikaryon of motor neurons (Côté et al., 1993; Xu et al., 1993; Lee et al., 1994). Overexpression of the human NFH gene in transgenic mice has been shown to cause motor

neuron degeneration (Côté et al., 1993) due to a reduction in NF transport and eventual interference with the axonal transport of other components such as actin, tubulin and mitochondria (Collard et al., 1995). The notion that NFs are directly involved in the etiology of ALS is also supported by the discovery of a small percentage of sporadic ALS cases with mutations in the KSP repeat domain of NFH (Figlewicz et al., 1994).

1.4 Rationale and objectives of the presented work

The objectives of the work presented in the following five chapters were: 1) to identify the neuronal IF subunits, protein kinase (s) and protein phosphatase (s) involved in neuronal IF dynamics, 2) to localize the phosphorylated amino acid residues responsible for neuronal IF disassembly, and 3) to determine the identity of kinase (s) involved in phosphorylation of the KSP repeats in NFH under both normal and stressful conditions. The rationale that guided experiments described in the next five chapters is outlined below:

1) Several studies reported that homopolymeric filaments composed of either NFL or α-internexin could be disassembled *in vitro* by phosphorylation of the head domain of the respective proteins (see section 1.3.5). However, there was a lack of information concerning the role of the other neuronal IF proteins in the assembly/disassembly process. It was also unclear whether head domain phosphorylation could regulate neuronal IF dynamics *in vivo*. The only compelling studies suggesting that phosphorylation could regulate neuronal IF assembly in living cells was performed by challenging cultured DRG neurons with the protein phosphatase inhibitors, okadaic acid and calyculin A. The latter treatments caused NF fragmentation with a concomitant increase in phosphorylation of the head domain of NFL. (Sacher et al., 1992; 1994). Initially, experiments were designed to determine which protein kinase and phosphatase activities could modulate NF assembly in cultured DRG neurons. Subsequently, I set out to determine whether the phosphorylation of specific amino acid residues in NFL, the key subunit in NF assembly, could be linked to the NF disassembly process in DRG neurons. Once antibodies became available, the studies were extended to study the fragmentation profiles and phosphorylation pattern of the other two neuronal IF proteins expressd in cultured DRG neurons, α-internexin and peripherin (Athlan et al., 1997).

2) α-internexin is the first neuronal IF protein expressed following neuronal diffentiation. Due to its early expression, it was speculated that the function α-internexin may be to form a more flexible neuronal IF network which may be required during

development of the nervous system (Nixon and Shea, 1992). I studied the expression and assembly of α-internexin and NF triplet proteins in the cerebral cortex to test the latter hypothesis. These studies were performed with neurons from cerebral cortex instead of DRGs since the former do not express peripherin and have a delayed onset of NF expression. The delay in NF expression provided a larger developmental time frame to correlate the assembly status of the different neuronal IF proteins with their expression patterns. The presence of only four neuonal IF proteins in the cerebral cortex, as compared to five in DRGs, made it easier to intepret the results.

3) Axonal NFH is one of the most highly phosphorylated proteins due to the presence of an extensively phosphorylated KSP repeat domain (see section 1.3.4). It is also known that perikaryal NFH is hypophosphorylated under normal conditions, and becomes highly phosphorylated in certain neurological diseases (Forno et al., 1986; Manetto et al., 1988; Zhang et al., 1989; Sobue et al., 1990; Pollanen et al., 1994) and following exposure to certain neurotoxins (Bizzi and Gambetti, 1986; Gold et al., 1988; Gold and Austin, 1991) or axotomy (Goldstein et al., 1987). During the course of NFH turnover studies in PC12 cells it was discovered that addition of N-acetyl-Leu-Leu-norleucinal (CI), a calpain and proteasome inhibitor, caused the hyperphosphorylation of NFH (Jayaraman et al., 1995). Based on this finding, I proceeded to show that CI also caused the hyperphosphorylation of perikaryal NFH in cultured DRG neurons. I then assessed different experimental approaches to identify the kinase(s) responsible for the CI-induced phosphorylation of NFH. I methodically eliminated the involvement of previously identified NFH-kinases and determined that novel members of the mitogen activated protein (MAP) kinase family, known as stress-activated protein kinases (SAPKs)(Cano and Mahadevan, 1995; Kyriakis and Avruch, 1996), are involved in aberrant phosphorylation of perikaryal NFH.

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Chapter 2

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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

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Abstract: The activation of cyclic AMP-dependent protein kinase (PKA) in rat dorsal root ganglion (DRG) cultures increased phosphorylation of the low-molecular-mass neurofilament subunit (NFL) at a site previously identified as Ser^{55} but had no effect on neurofilament integrity. When PKA was activated in DRG cultures treated with 20–250 n*M* okadaic acid, neurofilament fragmentation was enhanced, and there was a corresponding increase in phosphorylation of NFL at a novel site. This site was also phosphorylated by PKA in vitro and was determined to be Ser^{2} by mass spectrometric analysis of the purified chymotryptic phosphopeptide. The PKA sites in NFL were dephosphorylated by the purified catalytic subunit of protein phosphatase-2A but not that of protein phosphatase-1, and phosphoserine-2 was a better substrate than phosphoserine-55. The phosphorylation and dephosphorylation of Ser^{2} and Ser^{55} in NFL may therefore be involved in the modulation of neurofilament dynamics through the antagonistic effects of PKA and protein phosphatase-2A. **Key Words:** Neurofilament—Protein kinase A—Phosphatase—Phosphorylation—Okadaic acid.
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Mammalian neurofilaments (NFs) are composed of three type IV intermediate filament proteins with apparent molecular masses of 68 [low-(NFL)], 145 [middle-(NFM)], and 200 kDa [high-(NFH)] as determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Hoffman and Lasek, 1975; Fuchs and Weber, 1994). The predicted molecular masses of the rat NF subunits as deduced from their cDNA sequences are 61 [NFL (Chin and Liem, 1989)], 95 [NFM (Napolitano et al., 1987)], and 115 kDa [NFH (Chin and Liem, 1990)]. All three subunits are phosphoproteins (Julien and Mushynski, 1982), and the major phosphorylation sites of NFM and NFH are in their carboxy-terminal tail domain (Julien and Mushynski, 1983; Carden et al., 1985). The in vitro dephosphorylation of NFM and NFH causes the gel electrophoretic mobilities of both proteins to increase (Julien and Mushynski, 1982), indicating that their anomalous migration is in part due to phosphorylation.

NFs are known to be relatively stable structures, although they also appear to have certain dynamic properties. For example, an exchange of subunits between NFL filaments and a soluble pool of NFL has been reported (Angelides et al., 1989). The in vitro phosphorylation of NFL by either cyclic AMP (cAMP)-dependent protein kinase (PKA) or protein kinase C can block its polymerization or cause the disassembly of preexisting filaments (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990). Sihag and Nixon (1991) identified Ser^{55} of NFL as an in vivo PKA phosphorylation site, and they proposed that the cyclical phosphorylation and dephosphorylation of this site may regulate a step in NF assembly or transport. It is interesting to note in this regard that the catalytic subunit of PKA associates with NFs (Dosemeci and Pant, 1992).

Treatment of dorsal root ganglion (DRG) neurons with micromolar concentrations of okadaic acid (OA) has been reported to cause the fragmentation of NFs (Sacher et al., 1992). OA inhibits both protein phosphatase-2A (PP-2A; K_i = 0.2 n*M*) and protein phosphatase-1 (PP-1; K_i = 20 n*M*), although in the low nanomolar range it inhibits PP-2A more efficiently (Cohen et al., 1989; Ishihara et al., 1989). It has also been shown that PP-2A inhibition is at least partly

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Abbreviations used: cAMP, cyclic AMP; DRG, dorsal root ganglion; NF, neurofilament; NFH, NFL, and NFM, high-, low-, and middle-molecular-mass neurofilament subunit, respectively; OA, okadaic acid; PKA, cyclic AMP-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; PP-1 and PP-2A, protein phosphatase-1 and -2A, respectively; SDS, sodium dodecyl sulfate.

responsible for the fragmentation of NFs in OA-treated DRG cultures and that phosphorylation of the amino-terminal head domain of NFL increases under these conditions (Sacher et al., 1994).

In this study we show that the activation of PKA in DRG neurons treated with nanomolar concentrations of OA potentiated NF fragmentation and increased the phosphorylation of NFL at a novel site, Ser^2. This site is not located within a recognized consensus sequence for PKA and also serves as a substrate for PP-2A.

EXPERIMENTAL PROCEDURES

Materials

OA was purchased from LC Services (Woburn, MA, U.S.A.). N^6,2'-*O*-Dibutyryl cAMP, 8-bromo cAMP, forskolin, the catalytic subunit of PKA, and the PKA inhibitor protein (type III) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Highly purified PKA catalytic subunit was also purchased from Boehringer-Mannheim (Laval, Quebec, Canada). Carrier-free $^{32}P_i$ and [γ-^{32}P]ATP were from ICN Biomedicals (Mississauga, Ontario, Canada). Monoclonal antibodies (anti-NFL, NR4; anti-NFM, NN18; and anti-NFH, N52) were obtained from Sigma. Horseradish peroxidase-conjugated sheep anti-mouse IgG was obtained from Amersham. Texas Red-conjugated donkey anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). The catalytic subunits of PP-1 and PP-2A (PP-1_c and PP-$2A_c$, respectively) were purified from rabbit skeletal muscle following the procedure of Cohen et al. (1988).

Cell culture

Rat DRGs were dissected, dissociated, and maintained in defined medium as previously described (Sacher et al., 1992). For metabolic labeling cells were incubated with 0.25 mCi of carrier-free $^{32}P_i$/ml of P_i-reduced medium [19:1 of P_i-free medium (Flow Laboratories, McLean, VA, U.S.A.) to normal medium] for 3 h before treatment. Cells were treated with 2 m*M* dibutyryl cAMP for 3 h, 2 m*M* 8-bromo cAMP for 3 h, or 50 μ*M* forskolin for 12 h before OA treatment for 5 h.

Protein phosphatase and PKA treatment of NF subunits

An NF-enriched fraction was prepared from the white matter of rat brain and spinal cord (Georges et al., 1986), and the NF subunits were further purified using hydroxylapatite (Bio-Rad Laboratories) (Tokutake et al., 1983). The NF proteins were dialyzed against 5 m*M* Tris-HCl (pH 8.0), 0.1 m*M* EGTA, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 14.3 m*M* β-mercaptoethanol. NF subunits (0.2–1.0 mg/ml) were phosphorylated by incubating for 60 min at 30°C with 60 units/ml of the catalytic subunit of PKA, 0.5 m*M* [γ-^{32}P]ATP (50 μCi/μmol), 1 m*M* $MgCl_2$, 20 m*M* PIPES (pH 7), and 0.1 m*M* dithiothreitol. The reaction was stopped by adding 1 *M* guanidine-HCl, and the NF proteins were dialyzed against 65 m*M* Tris-HCl (pH 7), 0.5 m*M* EGTA, and 0.1 m*M* PMSF. The NFs were treated for 2 h at 30°C with or without PP-1_c/PP-$2A_c$ (1.5 μg/ml), PP-$2A_c$ (1.5 μg/ml), and OA in various combinations as described in the legend to Fig. 4.

Cell fractionation, gel electrophoresis, and western blotting

Cells were harvested in phosphate-buffered saline (137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, and 1.8 m*M* KH_2PO_4, pH 7.4) and lysed in Triton buffer [1% Triton X-100, 50 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl, 2 m*M* EGTA, 2 m*M* levamisole, 50 m*M* NaF, 1 m*M* PMSF, 25 μ*M* leupeptin, and 40 units/ml of aprotinin]. The extracts were centrifuged at 13,000 *g* for 30 min. Pellets and supernatants were solubilized in SDS sample buffer [50 m*M* Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 5% β-mercaptoethanol]. Gel electrophoresis was performed as previously described (Laemmli, 1970) using 5 or 7% polyacrylamide gels. Proteins were electrophoretically transferred to Immobilon-P membranes (Millipore Corp.) in buffer containing 48 m*M* Tris, 39 m*M* glycine, and 20% methanol. The membranes were blocked with 1% skimmed milk powder in Tris-buffered saline-Tween [20 m*M* Tris-HCl (pH 7.7), 137 m*M* NaCl, and 0.1% Tween-20], incubated with primary antibodies, and detected using the ECL Western Blotting Detection Kit (Amersham) following the manufacturer's instruction.

Immunofluorescence microscopy

Cells were stained with anti-NFL monoclonal antibody (NR4) as previously described (Sacher et al., 1992).

Immunoprecipitation

Cells were harvested in phosphate-buffered saline and lysed in 2% SDS and 50 m*M* Tris-HCl (pH 7.6) by boiling for 2 min. The SDS was diluted to 0.1% with Triton buffer. Immunoprecipitation was carried out as previously described (Lindenbaum et al., 1987) using a polyclonal rabbit antibody raised against NFL.

Phosphopeptide mapping

Following either immunoprecipitation of metabolically ^{32}P-labeled NFL from DRG cultures or phosphorylation of NFL in vitro, ^{32}P-labeled NFL resolved by gel electrophoresis was located by autoradiography and excised, and the gel slice was washed extensively with 20% methanol. Gel slices were lyophilized, and proteins were digested for 18 h at 37°C in 50 m*M* ammonium bicarbonate containing 20 μg/ml of N^{α}-*p*-tosyl-L-lysine chloromethyl ketone-α-chymotrypsin (Sigma). After the gel slices were removed, peptides were recovered by lyophilization and dissolved in water. Equal amounts of ^{32}P counts from each chymotryptic digest were loaded onto 20- × 20-cm cellulose sheets (MN-300, 0.1 mm thick; Brinkman, Westbury, NY, U.S.A.). Phosphopeptides were then resolved as described (Sihag et al., 1988) and were visualized by autoradiography.

Purification and analysis of phosphopeptide 2

Following the chymotryptic digestion of NFL, peptides were recovered by lyophilization, dissolved in 8% acetic acid, and applied to an AG 50W-X4 column (Bio-Rad Laboratories). Phosphopeptide 2 (see Fig. 5) was recovered in the flow-through, lyophilized, and further purified on a C-18 reverse-phase column (Vydac, Hesperia, CA, U.S.A.). The peptide was resolved with a 0–80% gradient of acetonitrile in 0.12% trifluoroacetic acid. Each purification step was monitored by two-dimensional mapping (Sihag et al., 1988) to verify the presence of phosphopeptide 2. Amino acid and sequence analyses of phosphopeptide 2 were performed at the Harvard Microchemistry Facility (Cambridge, MA,

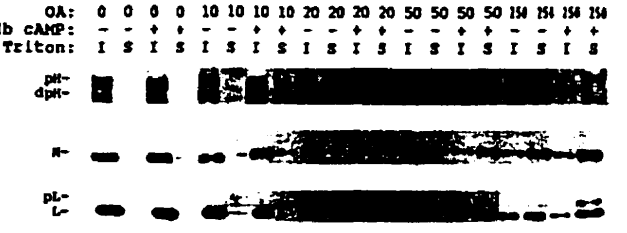

FIG. 1. Western blot analysis of the effects of dibutyryl cAMP (db cAMP) on NFs in DRG neurons treated with increasing concentrations of OA. DRG cultures were fractionated into Triton X-100-insoluble (I) and -soluble (S) fractions by centrifugation at 13,000 *g* for 30 min. Samples were resolved by SDS-polyacrylamide gel electrophoresis, and the NF subunits were detected by western blotting as described in Experimental Procedures. The OA concentrations indicated above each lane were in nanomolar. Cultures were either pretreated (+) or not treated (−) with 2 m*M* db cAMP. pH, M, and L refer to the native NF subunits. dpH refers to hypophosphorylated NFH, and pL refers to a more highly phosphorylated form of NFL.

U.S.A.). Sequence analysis was determined by collisionally activated dissociation on a Finnigan TSQ triple quadrupole mass spectrometer.

RESULTS

Activation of PKA enhances the fragmentation of NFs in OA-treated DRG neurons

The reversible fragmentation of NFs has been observed in cultured DRG neurons treated with OA (Sacher et al., 1992). This fragmentation, which was assessed by monitoring the solubility of all three NF subunits in Triton X-100 at 13,000 *g*, was increased by pretreating the cultures with dibutyryl cAMP, an activator of PKA. There was no significant increase in the Triton X-100 solubility of NFs when dibutyryl cAMP was added without OA or with 10 n*M* OA for 5 h. However, at OA concentrations of ≥20 n*M*, addition of dibutyryl cAMP caused a significant increase in the Triton X-100 solubility of all three NF subunits (Fig. 1). Two other activators of PKA, 8-bromo cAMP and forskolin, also enhanced the fragmentation of NFs in the presence of 20 n*M* OA (Fig. 2). The observed disruption of NFs by 20 n*M* OA in the presence of dibutyryl cAMP supports previous evidence (Sacher et al., 1994) indicating that the disruption of NFs in OA-treated neurons is mostly due to the inhibition of PP-2A rather than PP-1.

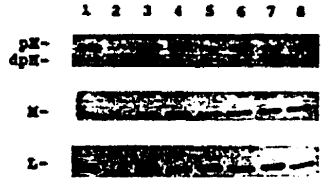

FIG. 2. Western blot analysis of NF subunits in DRG cultures treated with OA and different activators of PKA. DRG cultures treated with 20 n*M* OA were fractionated into Triton-insoluble (odd-numbered lanes) and -soluble fractions (even-numbered lanes). The samples were analyzed as in Fig. 1. Cultures were also pretreated with 2 m*M* dibutyryl cAMP (lanes 3 and 4), 2 m*M* 8-bromo cAMP (lanes 5 and 6), or 50 μ*M* forskolin (lanes 7 and 8). The cells analyzed in lanes 1 and 2 were treated with OA only. The abbreviations used to label this figure are defined in the legend to Fig. 1.

The increase in Triton X-100 solubility of NFs seen when PKA was activated in the presence of OA correlated with a disruption of the organization of NFs as seen by immunofluorescence microscopy (Fig. 3). The neurites of untreated neurons showed a smooth, continuous NF staining pattern (Fig. 3A), and addition of dibutyryl cAMP alone (Fig. 3B) or 20 n*M* OA alone (Fig. 3C) did not produce a significant change in NF distribution. Minor levels of NF disruption began to appear with 50 n*M* OA (Fig. 3E). Pretreatment of cell cultures with dibutyryl cAMP caused a total collapse of the NF network in the presence of either 20 (Fig. 3D) or 50 (Fig. 3F) n*M* OA. The NF staining pattern in the perikaryon of DRG neurons was not altered by these treatments, although the cell bodies of neurons exposed to 50 n*M* OA and dibutyryl cAMP tended to round up.

PP-2A dephosphorylates NFL and NFM that have been phosphorylated by PKA

NFL and NFM phosphorylated by PKA in vitro were dephosphorylated by PP-$2A_c$ (Fig. 4, lane 2), and this activity was totally inhibited by 10 n*M* OA (Fig. 4, lane 3). A preparation that contained both PP-$2A_c$ and PP-1_c also dephosphorylated NFL and NFM that had been phosphorylated by PKA (Fig. 4, lane 5), although this activity could be completely inhibited by 10 n*M* OA (Fig. 4, lane 6). This concentration of OA is sufficient to inhibit completely PP-2A but not PP-1 (Cohen et al., 1989; Ishihara et al., 1989), again indicating that PP-$2A_c$ and not PP-1_c was responsible for the observed dephosphorylation of the two NF subunits.

The augmentation in NF fragmentation due to PKA activation correlates with the phosphorylation of NFL at a novel site

Rat NFL can be phosphorylated by PKA in vitro at two sites designated as 1 and 2 in the chymotryptic phosphopeptide map shown in Fig. 5A. Phosphopeptide 1 probably corresponds to peptide L2 of Sihag and Nixon (1991) because the two show similar mobilities when resolved by the same two-dimensional mapping

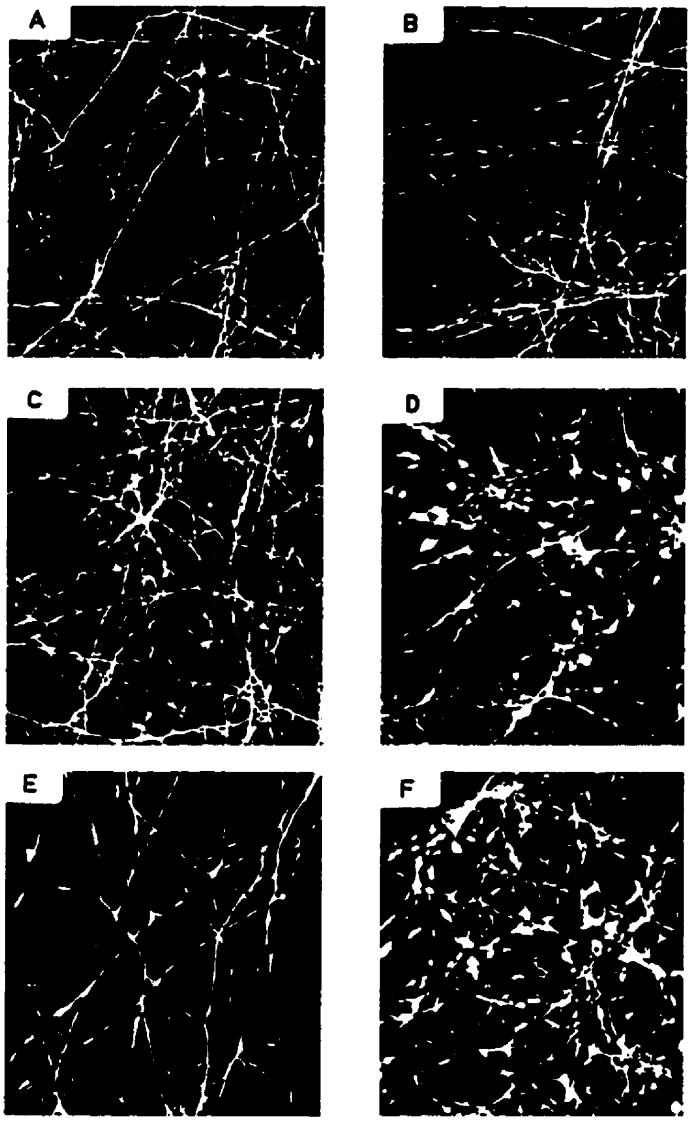

FIG. 3. Immunofluorescence microscopy of OA- and dibutyryl cAMP-treated DRG cultures. DRG cultures were untreated (A and B), or treated with 20 n*M* OA (C and D) or 50 n*M* OA (E and F). B, D, and F were pretreated with 2 m*M* dibutyryl cAMP. The cells were stained with an anti-NFL monoclonal antibody as described in Experimental Procedures.

procedure. This peptide contains Ser^{55}, a major PKA phosphorylation site in mouse NFL (Sihag and Nixon, 1991), and has the same sequence in rat NFL (Chin and Liem, 1989). Phosphopeptide 1 was also phosphorylated when DRG cultures were treated with a PKA activator (cf. Fig. 5C and E). Phosphorylation of phosphopeptide 2 in NFL from DRG cultures treated with dibutyryl cAMP (Fig. 5C) or 50 n*M* OA (Fig. 5D) was barely detectable, whereas combined treatment with both OA and dibutyryl cAMP increased its phosphorylation dramatically (Fig. 5E). A similar synergistic effect on the phosphorylation of site 2 was obtained with 20 n*M* OA and dibutyryl cAMP (data not shown). Site 2 was also a better substrate than site 1 for in vitro dephosphorylation by PP-$2A_c$ (Fig. 6), perhaps explaining why OA is required to obtain high levels of phosphopeptide 2 in vivo.

Identification of the novel PKA phosphorylation site in NFL

Phosphopeptide 2 shown in Fig. 5 has unusual electrophoretic properties. The buffer used for electrophoresis was at pH 1.5, where carboxylic groups and phosphate groups have a greatly reduced negative charge whereas amino groups have a full positive charge. Consequently, peptides normally migrate toward the cath-

FIG. 4. Gel electrophoretic analysis of NFL and NFM phosphorylated with PKA and treated with PP-1_c and PP-$2A_c$. NFs were phosphorylated with PKA and treated with PP-1_c and PP-$2A_c$ as described in Experimental Procedures. Lane 1 of the autoradiograph shows NFL and NFM not treated with phosphatase. In lanes 2, 3, and 4, NFs were incubated with PP-$2A_c$. In lanes 5, 6, and 7, NFs were incubated with a preparation containing both PP-1_c and PP-$2A_c$. OA was also added to the reaction at a concentration of 10 n*M* (lanes 3 and 6) and 1 μM (lanes 4 and 7).

ode at this pH. The fact that phosphopeptide 2 migrated toward the anode, did not bind to the cation exchange resin (AG 50W-X4), and could not be N-terminally sequenced suggested that its N-terminal amino group was blocked.

The phosphopeptide 2 used for characterization was derived from chymotryptic digests of NFL that was phosphorylated by PKA in vitro. An average of ~0.25 mol of phosphate/mol of NFL was incorporated in different experiments, and equivalent amounts of ^{32}P were incorporated into phosphopeptides 1 and 2 (Fig. 5A). The same results were obtained with PKA catalytic subunit purchased from two different sources and the in vitro reaction was completely abolished by the Walsh inhibitor (Walsh et al., 1971) of PKA (data not shown). Phosphoamino acid analysis showed that phosphopeptide 2 contained phosphoserine (data not shown). Amino acid composition analysis of the peptide revealed that it most likely contained 2 mol of Ser and 1 mol of Phe/mol of peptide (Table 1). Chymotryptic digestion of NFL should produce a peptide from the amino-terminus with the sequence *N*-acetyl-Ser-Ser-Phe, which would have the properties observed for phosphopeptide 2. The calculated mass of 461.4 for phosphopeptide 2 containing a single phosphate moiety was verified by mass spectrometric analysis, which yielded a value of 461.0. The high confidence sequence *N*-acetyl-Ser-Ser(PO_4)-Phe was determined by collisionally activated dissociation.

DISCUSSION

The NF fragmentation that is induced by treatment of DRG neurons with OA may be due to the trapping of intermediate species involved in NF dynamics. The OA-induced fragmentation is reversible up to a point (Sacher et al., 1992) and is due at least in part to the inhibition of PP-2A (Sacher et al., 1994). The results presented in this report indicate that PKA is also involved in this process as NF fragmentation is markedly enhanced when this kinase is activated in neurons treated with low concentrations of OA.

There are several reasons why Ser^2 of NFL may have been overlooked as a PKA phosphorylation site in the head domain at the time that phosphorylation of Ser^{55} was established (Sihag and Nixon, 1991). Most significant is that, the anomalous electrophoretic mi-

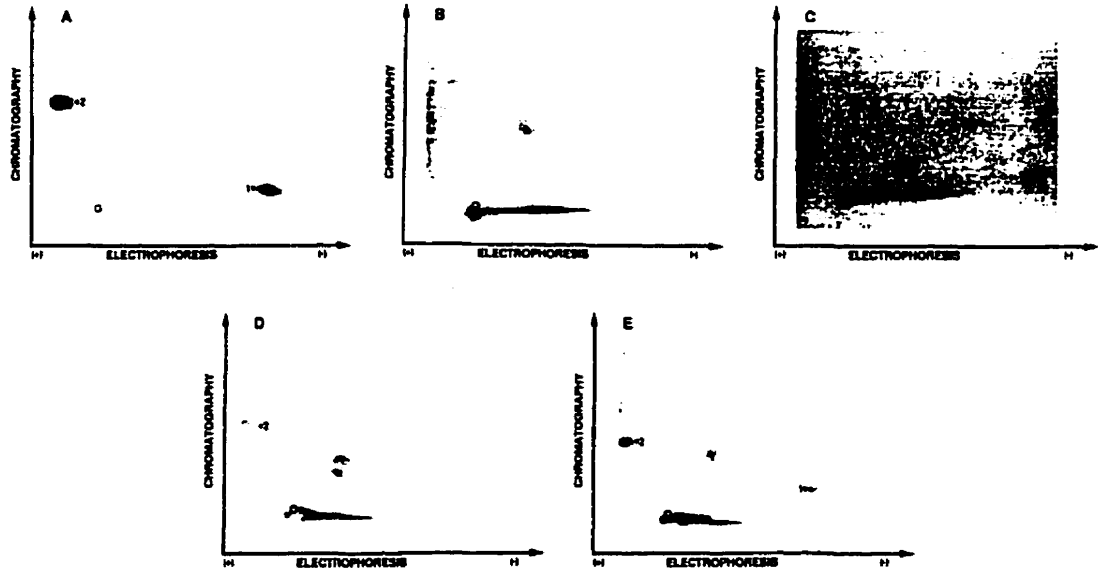

FIG. 5. Two-dimensional phosphopeptide mapping of NFL. A: Rat NFL was phosphorylated with PKA in vitro, resolved by SDS-PAGE, and subjected to two-dimensional phosphopeptide mapping as described in Experimental Procedures. DRG cultures were preincubated with ^{32}P, and left untreated (B) or were treated with dibutyryl cAMP (C), 50 n*M* OA (D), or dibutyryl cAMP plus 50 n*M* OA (E), and immunoprecipitated NFL was mapped as described in Experimental Procedures. O represents the origin, and the numbers 1 and 2 indicate the two NFL peptides phosphorylated by PKA.

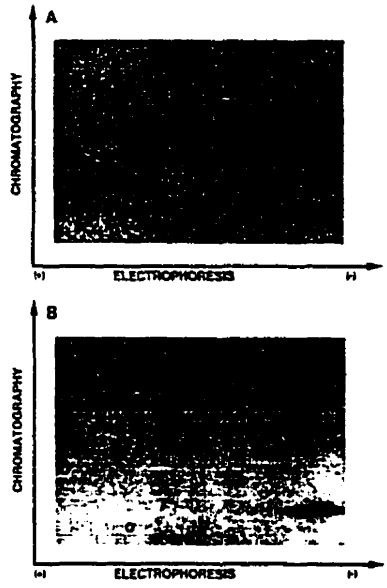

FIG. 6. Two-dimensional phosphopeptide mapping of NFL dephosphorylated by PP-2A$_C$. Rat NFL was phosphorylated by PKA in vitro and left untreated (A) or further subjected to dephosphorylation by PP-2A$_C$ (B) as described in Experimental Procedures. Following chymotryptic cleavage, equal amounts of radioactivity were loaded. O represents the origin, and 1 and 2 denote the two NFL peptides phosphorylated by PKA.

gration of phosphopeptide 2 increases the likelihood that it will not be detected because it migrates toward the anode at pH 1.5 and can run off the cellulose sheet during electrophoresis. In addition, NFL is N-terminally blocked (Geisler et al., 1983), making it impossible to sequence the N-terminal chymotryptic peptide by conventional means. Phosphopeptide 2 does not appear to correspond to peptide L3 from previous reports (Sihag and Nixon, 1989, 1991), as the latter migrated toward the cathode under the same conditions used here. Peptide L3 was also shown to undergo radioiodination, indicating that it contains Tyr (Sihag and Nixon, 1989).

Phosphopeptide mapping of mouse and bovine NFL phosphorylated with PKA in vitro showed them to be phosphorylated at the same two sites as were seen for rat NFL in Fig. 5A (data not shown). The identification of Ser^2 as a phosphorylation site in the head domain of NFL agrees with studies reporting that bovine NFL treated with high levels of PKA could be phosphorylated to the extent of 2–3 mol of phosphate/mol of polypeptide (Hisanaga et al., 1990; Nakamura et al., 1990). In addition, a recent report suggested the existence of a PKA site within the first 14 amino acids of porcine NFL, a region that does not contain a consensus PKA phosphorylation site (Hisanaga et al., 1994). Because the amino-terminal tripeptide of NFL similarly does not contain a PKA consensus sequence, Ser^2 may be in a context where proximal basic amino acids from a noncontiguous sequence specify phosphorylation (Kennelly and Krebs, 1991). Of >100 PKA phosphorylation sites reported in the literature, this appears to be the first not to have a basic amino acid to the N-terminal side of the site.

The changes in NF distribution seen in neurons treated with OA and activators of PKA appear to involve a shift in equilibrium between the antagonistic effects of PP-2A and PKA. The phosphorylation of Ser^2 of NFL may be directly involved in causing an increase in NF fragmentation. This site is a substrate for PKA and PP-2A in vitro, and its in vivo phosphorylation state appears to be regulated by these two enzymes. Because Ser^{55} is phosphorylated along with Ser^2 under conditions that augment NF fragmentation, both sites may be involved in this process.

The limited fragmentation of NFs that occurred in neurons treated with 50 n*M* OA alone did not appear to be paralleled by a significant increase in the phosphorylation of NFL (Fig. 5D). The low level of Ser^2 phosphorylation seen under these conditions was approximately the same as in neurons treated with dibutyryl cAMP alone, which did not cause NF fragmentation. The reason for this apparent discrepancy is unknown at present. Perhaps these two systems differ at some other level, such as the extent of NFM or NFH phosphorylation.

The notion that phosphorylation of the amino-terminal head domain of NFL is involved in the fragmentation of NFs is supported directly by evidence that NFL homopolymers can be disassembled by PKA treatment in vitro (Hisanaga et al., 1990; Nakamura et al., 1990) and indirectly by the results presented in this report. On the other hand, treatment of native NFs with PKA in vitro did not cause disassembly (Hisanaga et al., 1994), suggesting that other factors may be involved or that the properties of NFs may be altered during the course of their isolation.

The changes in NF distribution seen in DRG neurons

TABLE 1. *Amino acid composition analysis of phosphopeptide 2*

Amino acid	Yield (pmol)
Ser	61.0[a]
Phe	40.6
Gly	13.3
Glx	7.7
Leu	4.0
Ala	3.1
Ile	3.1

Phosphopeptide 2 was purified as described in Experimental Procedures, and 50 pmol of the peptide as determined from its ^{32}P specific radioactivity was subjected to amino acid composition analysis.

[a] This value is an underestimation because *O*-phosphoserine is inefficiently converted to Ser by acid hydrolysis (Glazer et al., 1976).

treated with OA and a PKA activator appear to involve a shift in the equilibrium between the antagonistic effects of PP-2A and PKA. These two enzymes thus appear to be involved in modulating the dynamic properties of NFs. Defining the various components involved in modulating NF dynamics may provide some insight into mechanisms underlying the aberrant aggregation of NFs in certain neurological disorders.

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Chapter 3

Intermediate Filament Disassembly In Cultured Dorsal Root Ganglion Neurons Is Associated With Amino-Terminal Head Domain Phosphorylation Of Specific Subunits

Abstract

We previously reported that activation of protein kinase A in cultured rat dorsal root ganglion neurons, treated concomitantly with low concentrations of okadaic acid that selectively inhibit protein phosphatase-2A, enhanced the Triton X-100 solubility of neurofilament triplet proteins (Giasson et al., 1996). We now show that peripherin and α-internexin follow the same fragmentation profile as the neurofilament subunits, consistent with the notion that all five cytoplasmic intermediate filament proteins in these neurons form an integrated filamentous network whose assembly can be modulated by protein kinase A. Similar to the situation previously observed for the low molecular mass neurofilament subunit (NFL), there was a strong correlation between phosphorylation of the amino-terminal head domain of peripherin and filament fragmentation. On the other hand, insignificant levels of ^{32}P were incorporated into α-internexin under conditions promoting disassembly, indicating that phosphorylation of this protein is not directly involved in filament fragmentation. The situation for the mid-sized neurofilament subunit (NFM) was not as clear-cut. Phosphopeptide mapping of NFM revealed many head and tail domain phosphorylation sites . However, changes in NFM head domain phosphorylation under conditions promoting filament disassembly were not as pronounced as for peripherin.

Introduction

The intermediate filament (IF) network in cultured embryonic dorsal root ganglion (DRG) neurons is composed of five cytoplasmic intermediate filament (IF) proteins. They include the neurofilament (NF) triplet proteins, NFL, NFM and NFH, together with peripherin and α-internexin (Goldstein et al., 1996; Athlan et al., 1997). These neuronal IF proteins share a domain organization common to all IF proteins, consisting of an α-helical rod domain flanked by amino-terminal head and carboxyl-terminal tail domains (for a review see Fuchs and Weber, 1994). IFs in different cell types also display a dynamic behavior that is at least partly modulated by phosphorylation/dephosphorylation (for reviews see Skalli and Goldman, 1991; Nixon and Shea, 1992).

Evidence that phosphorylation plays a role in NF dynamics is particularly compelling in the case of NFL, the key subunit in NF assembly (Ching and Liem, 1993; Lee et al., 1993; Ohara et al., 1993). The in vitro phosphorylation of NFL by either cyclic AMP-dependent protein kinase (PKA) or protein kinase C affects homopolymer assembly (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990). Phosphorylation sites affecting NFL assembly are located in the head domain (Sihag and Nixon, 1989; Sacher et al., 1994), where Ser-55 was initially identified as a PKA site likely to be involved in NF dynamics (Sihag and Nixon, 1991). A second PKA site in the head domain of NFL, Ser-2, was identified in a study involving DRG neurons (Giasson et al., 1996). The latter study also demonstrated that NF assembly in DRG neurons can be modulated through the antagonistic effects of PKA and protein phosphatase (PP)-2A.

Evidence that any of the other four neuronal IF proteins are similarly modified during filament fragmentation is less compelling, although in vitro studies suggest that phosphorylation of NFM (Streifel et al., 1996) and α-internexin (Tanaka et al., 1993) may be similarly involved in modulating filament assembly. The only evidence linking peripherin phosphorylation with assembly/disassembly suggests that in PC12 cells highly phosphorylated peripherin is more stably associated with the cytoskeletal network (Aletta et al., 1989).

Current knowledge concerning the phosphorylation of α-internexin and peripherin in vivo is very limited. Peripherin has been shown to be phosphorylated in PC12 cells, NIE 115 cells and sympathetic neurons (Aletta et al., 1989; Huc et al., 1989) and its phosphorylation in the two latter cell types is largely restricted to the head domain (Huc et

al., 1989). On the other hand, nothing is known about the in vivo phosphorylation state of α-internexin.

The IF cytoskeleton in cultured DRG neurons is a highly integrated network (Athlan et al., 1997). It was therefore of interest to determine whether other neuronal IF components show a correlation between head domain phosphorylation and IF fragmentation similar to that seen for NFL (Giasson et al., 1996). The approach we used consisted of treating DRG cultures with low concentrations of the PP-2A and PP-1 inhibitor, okadaic acid (OA) (Cohen et al., 1989), in the presence or absence of a PKA activator (Giasson et al., 1996). Chemical cleavage techniques and comparative phosphopeptide mapping of various neuronal IF proteins revealed a strong correlation between phosphorylation and filament disassembly for peripherin and a lesser one for NF-M. α-internexin did not appear to be involved in this process, since extremely low levels of ^{32}P were incorporated into the protein under conditions promoting filament fragmentation.

Experimental Procedures

Materials

OA was purchased from LC Services (Woburn, MA). N^6,2'-O-Dibutyryl cyclic AMP (db cAMP), the catalytic subunit of PKA, anti-mouse IgG_1 antibody-agarose and monoclonal anti-NFL antibody (NR4) were from Sigma Chemical Co. (St. Louis, MO). Carrier-free $^{32}P_i$ and [γ-^{32}P]-ATP were from ICN Biomedicals (Mississauga, ON). Monoclonal anti-peripherin (MAB 1527) and anti-α-internexin (MAB1525) antibodies were purchased from Chemicon International (Temecula, CA). Enhanced chemiluminescence reagents were from NEN (Mississauga, ON). Protein A-Sepharose was obtained from Pharmacia Biotech (Baie D'Urfé, PQ)

Cell culture

Rat DRGs and cerebral cortex were dissected, dissociated and maintained in culture for 20 days and 5 days, respectively, as described previously (Giasson and Mushynski 1996; 1997). For metabolic labelling cells were incubated with 0.25 mCi of carrier-free $^{32}P_i$ /ml of P_i-reduced medium [19:1 of P_i-free medium (Flow Laboratories, McLean, VA)

to normal medium] for 3 h before treatment. Cells were treated with 2 mM db cAMP for 3h before the addition of OA for 5 h.

Cell fractionation, gel electrophoresis, and western blotting

Cells were harvested in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and lysed in Triton buffer [1% Triton X-100, 50 mM Tris (pH 7.5), 100 mM NaCl, 2 mM EGTA, 2 mM levamisole, 50 mM NaF, 1 mM PMSF, 25 μM leupeptin, and 40 units/ml aprotinin]. The extracts were centrifuged at 13,000xg for 30 min. Pellets and supernatants were solubilized in sodium dodecyl sulfate (SDS) sample buffer [50 mM Tris (pH 6.8), 10% glycerol, 2% SDS and 5% β-mercaptoethanol]. Gel electrophoresis was performed as previously described (Laemmli, 1970). For western blotting, proteins were electrophoretically transferred to Immobilon-P membranes (Millipore Corp.) in buffer containing 48 mM Tris, 39 mM glycine and 10% methanol. The membrane was blocked with 1% skimmed milk powder dissolved in Tris-buffered saline-Tween [20 mM Tris (pH 7.7), 137 mM NaCl and 0.1% Tween-20], incubated with primary antibodies and visualized by enhanced chemiluminescence as described by the manufacturer.

Immunoprecipitation

Cells were harvested in phosphate-buffered saline and lysed in detergent buffer [150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 20 mM NaF, 2 mM EGTA, 0.5% levamisole, 1 mM $NaVO_4$, 1mM PMSF, 25 μM leupeptin and 40 units/ml aprotinin]. Cellular debris was removed by centrifugation at 13,000xg for 30 sec. and 1% SDS was added to the supernatant. Samples were boiled for 5 min. and diluted 10-fold in Triton buffer. The proteins of interest were immunoprecipitated for 3 h at 4°C with either a polyclonal rabbit antibody raised against NFM bound to protein A-Sepharose, or anti-peripherin or anti-α-internexin antibodies bound to anti-mouse IgG_1 antibody-agarose beads. The immunoprecipitates were washed repeatedly with Triton buffer and eluted by boiling for 5 min in SDS sample buffer.

Chemical cleavage of peripherin

Immunoprecipitated, ^{32}P-labelled peripherin was resolved by SDS-PAGE in the presence of 2 mM thioglycolic acid. The gel was washed in 20% methanol and dried. The peripherin band was located by autoradiography and excised. The gel slice was rehydrated in N-chlorosuccinimide (NCS) buffer (1 g urea, 1 ml H_2O and 1 ml acetic acid)(Lischwe and Ochs, 1982) and incubated at room temperature for 1.5 h in the same buffer containing 2 mg/ml NCS. The slices were washed with H_2O, equilibrated in SDS sample buffer and resolved by SDS-PAGE.

Phosphorylation of NFM with PKA in vitro

An NF-enriched fraction was prepared from rat brain stem and spinal cord (Georges et al., 1986), and the NF subunits were further purified using hydroxylapatite (Bio-Rad Laboratories)(Tokutake et al., 1983). The NF proteins were dialyzed against 5 mM Tris (pH 8.0), 1 mM EGTA, 0.1 mM PMSF and 14.3 mM β-mercaptoethanol. NF subunits were phosphorylated by incubation for 60 min at 30°C with 60 units/ml of the catalytic subunit of PKA, 0.1 mM [γ-^{32}P]-ATP (50 μCi/μM), 1 mM $MgCl_2$, 20 mM PIPES (pH 7.0) and 0.1 mM dithiothreitol. The reaction was stopped with the addition of SDS sample buffer.

Phosphopeptide mapping

Following either immunoprecitation of metabolically ^{32}P-labelled proteins or phosphorylation of NFM in vitro, the proteins were resolved by SDS-PAGE, visualized by autoradiography and excised from the gel. The gel slices were washed with 20% methanol and lyophilized. Peripherin and NFM were digested for 18 h at 37°C in 50 mM ammonium bicarbonate containing 10 μg/ml of N^{α}–p-tosyl-L-lysine chloroketone-treated α-chymotrypsin (Sigma). NFM was further digested with 10 μg/ml trypsin (Sigma) for 6 h at 37°C. After the gel slices were removed, peptides were lyophilized and dissolved in H_2O and loaded onto cellulose TLC sheets (MN-300; EM Science, Gibbstown, NJ). Phosphopeptides were resolved as described (Sihag and Nixon, 1990) and were visualized by autoradiography.

Results

In a previous study we had shown that an increase in the Triton solubility of NF subunits in cultured DRG neurons correlated with phosphorylation of two PKA sites in the head domain of NFL (Giasson et al., 1996). It was subsequently shown that two additional IF proteins, peripherin and α-internexin, formed a highly integrated network together with the NF triplet proteins in these neurons (Athlan et al., 1997). The question therefore arose as to whether α-internexin and peripherin followed the same PKA-dependent fragmentation pattern as the NF triplet proteins. Fig. 1 shows a Western blot analysis of Triton-insoluble and Triton-soluble fractions from DRG neurons treated with nM concentrations of okadaic acid in the presence or absence of the PKA activator, db cAMP. Treatment with db cAMP or 20 nM OA alone did not significantly increase the levels of Triton-soluble NFL, α-internexin or peripherin. Roughly half of each IF protein was rendered Triton-soluble when neurons were treated with db cAMP and 20 nM OA together. Higher levels of solubilization were achieved when PKA was activated in the presence of 50 nM OA.

PKA and OA increased the Triton-solubility of all five neuronal IF proteins in DRG neurons (Fig. 1 and Giasson et al., 1996), indicating that phosphorylation sites were being targetted to promote filament disassembly. We had already demonstrated that fragmentation of neuronal IFs correlated with phosphorylation of Ser residues in the head domain of NFL (Giasson et al., 1996). We therefore proceeded to determine the phosphorylation status of other IF proteins in DRG neurons under conditions promoting filament fragmentation. Fig. 2 shows that db cAMP or 50 nM OA alone increased the ^{32}P-labelling of peripherin, although the highest level of ^{32}P-labelling was achieved in neurons treated with both compounds together. The ^{32}P-label in peripherin was located predominantly in the N-terminal 32.5 kDa polypeptide (Fig. 3). The phosphorylation sites in this polypeptide are most probably in the head domain, since it is highly unlikely that the α-helical rod segment is phosphorylated (Steinert et al., 1982).

Fig. 4A shows that peripherin from untreated DRG neurons contains one major chymotryptic phosphopeptide (phosphopeptide 1) as well as several minor species, some of which become more prominent following activation of PKA and/or phosphatase inhibition. These phosphopeptides can be grouped in three categories: those that become more prominent upon PKA activation (4 and 5 in Fig. 4B) or with OA treatment (4-9 in Fig. 4C), as well as species that become even more prominent when neurons are treated with both OA and db cAMP (4, 5, 10 and 11 in Fig. 4D). Whether the latter four sites are

especially crucial in promoting filament fragmentation is still a matter of conjecture because of the large number of phosphorylation sites in the peripherin head domain. However, the clear-cut synergism between OA and db cAMP strongly supports this notion.

Previous work (Sihag and Nixon, 1990; Sihag et al., 1995) revealed two classes of phosphorylation sites in NFM: sites in the head domain phosphorylated by PKA and/or protein kinase C, and tail domain sites phosphorylated by second messenger-independent NF kinases. The phosphopeptide map in Fig. 5A was derived from NFM that had been phosphorylated by PKA in vitro and digested by chymotrypsin and trypsin. These sites have been shown to be located in the head domain (Sihag and Nixon, 1990; Sihag et al., 1995). With the possible exception of phosphopeptide 2, the phosphorylation of these phosphopeptides was not detected under control conditions (Fig. 5B). However, the latter did contain prominent tail domain phosphopeptides as indicated by the sites labelled with asterisks (Sihag and Nixon, 1990). The phosphopeptide map of NFM from db cAMP-treated neurons contained head domain phosphopeptides 2, 4, 5 and 8 (Fig. 5C), illustrating that these PKA sites can be phosphorylated in vivo. Treating the neurons with OA caused the appearance of a single novel species, phosphopeptide 1 (Fig. 5D). Treatment of DRG neurons with OA and db cAMP combined (Fig. 5E) did not result in any outstanding changes in the phosphopeptide map compared to that seen with either agent alone, but it did cause an increase in the phosphorylation of phosphopeptide 1. Unless combined phosphorylation of phosphopeptides 1 and 2 is particularly crucial for filament disassembly, these results make it difficult to decide whether head domain phosphorylation in NFM plays a significant role in the fragmentation process.

α-internexin has been shown to undergo head domain phosphorylation by PKA in vitro (Tanaka et al., 1993). However, metabolic ^{32}P-labelling studies demonstrated that α-internexin immunoprecipitated from DRG cultures contained insignificant levels of ^{32}P-label (Fig. 6A, lanes 1-4), even under conditions promoting filament fragmentation. In contrast, α-internexin from cultured cortical neurons, where it is the major neuronal IF protein (Giasson and Mushynski, 1997), showed some ^{32}P-labelling in OA-treated cultures (Fig. 6A, lane 7). The autoradiograms of immunoprecipitated α-internexin (Fig. 6) and peripherin (Fig. 2) were exposed for 168 h and 12 h, respectively. Since western blots of the immunoprecipitates indicated that approximately equal amounts of the two proteins were present, these results demonstrated that insignificant amounts of ^{32}P were incorporated into α-internexin even when DRG neurons were treated with db cAMP and/or 50 nM OA.

Discussion

We recently demonstrated that PKA activation and OA had a synergistic effect on increasing the Triton solubility of NF triplet proteins in cultured DRG neurons (Giasson et al., 1996). The present report shows that under the same conditions the increase in Triton solubility of peripherin and α-internexin paralleled that of the NF subunits. This finding is consistent with the notion that IFs in DRG neurons form a highly integrated network (Athlan et al., 1997) and indicates that neuronal IF dynamics are modulated by mechanisms shared with other types of IFs (Skalli and Goldman, 1991; Nixon and Shea, 1992).

Analysis of NFL phosphorylation in DRG neurons revealed a relatively simple phosphopeptide map, involving two head domain sites, Ser-2 and Ser-55 (Sihag and Nixon, 1991; Giasson et al., 1996) and tail domain phosphorylation probably occurring mainly at Ser-473 (Sihag and Nixon, 1989; Xu et al., 1990). We had also reported that the phosphorylation of Ser-2 correlated with neuronal IF fragmentation. The situation for peripherin is more difficult to interpret because phosphopeptide mapping revealed a large number of phosphorylation sites (Fig. 4), which were virtually completely restricted to the head domain under all of the conditions tested here (Fig. 3). In cells treated with db cAMP and OA combined, four peripherin phosphopeptides, including the two that contained PKA sites, were more highly ^{32}P-labelled compared to the corresponding species from neurons treated with either agent alone. These 4 sites, perhaps acting synergistically with other sites phosphorylated in the presence of db cAMP or OA alone, are likely to be involved in disassembly of the IF network in DRG neurons.

The essential role of OA in achieving significant levels of IF fragmentation further illustrates the key role of protein phosphatases in maintaining cytoskeletal integrity. OA inhibits both PP-1 and PP-2A, although it is a more potent inhibitor of PP-2A (Cohen et al., 1989). In view of the low concentrations of OA employed in this study, PP-2A appears to be the phosphatase involved in preventing neuronal IF fragmentation. It has been demonstrated that 100 nM OA had little effect on the intracellular activity of PP-1, while 30-100 nM of the same compound significantly inhibited PP-2A (Favre et al., 1997). It is interesting to note in this regard that PP-2A is associated with and may be involved in preserving NFs (Saito et al., 1995).

The presence of second messenger dependent protein kinase sites in the head domain of NFM had led to the suggestion that they may play a role in NF dynamics (Sihag and Nixon, 1990). The differences between the phosphopeptide maps of NFM from db cAMP and OA-treated neurons were relatively minor with the notable exception of species

1 and 2 (cf. Figs. 5C and 5D). Whether phosphorylation at these two sites together in neurons treated with both db cAMP and OA is at least partly responsible for the marked increase in IF fragmentation is still a matter of conjecture. Perhaps filament fragmentation is due to the cumulative effect of head domain phosphorylation in the different subunit proteins, excepting α-internexin, that make up the neuronal IF network in DRG neurons.

Previous studies demonstrated that in vitro phosphorylation by PKA of head domain sites in α-internexin blocked its ability to polymerize (Tanaka et al., 1993). The lack of α-internexin phosphorylation in DRG neurons under conditions causing IF fragmentation was therefore quite unexpected. The possibilty was considered that head domain sites in α-internexin were occluded as a result of its coassembly with the other four neuronal IF proteins in DRG neurons (Athlan et al., 1997). However, phosphorylation of α-internexin in cultured cortical neurons was also exceedingly low (Fig. 6) despite the fact that it is the major IF protein in these neurons (Giasson and Mushynski, 1997).

The low levels of α-internexin phosphorylation observed even in the presence of db cAMP and OA, suggests that this IF subunit is not directly involved in fragmentation mediated by phosphorylation. However, a recent study suggests that this protein may be involved in forming a more flexible IF network (Giasson and Mushynski, 1997). The presence of α-internexin in the neuronal IF network of cultured embryonic DRG neurons may thus render these IFs more responsive to the effects of head domain phosphorylation.

Our findings clearly indicate that both PKA and PP-2A can be involved in modulating the dynamics of the highly complex IF network present in cultured DRG neurons. The phosphorylation of sites in the head domains of NFL, NFM and peripherin may be linked with this process. It remains to be determined which and how many of these phosphorylation sites are actually involved in promoting IF disassembly, although such an undertaking would require the prior identification of those sites that have not yet been characterized. Even though extensive cytoskeletal rearrangements such as that caused by db cAMP and OA treatment (see also, Giasson et al., 1996) are unlikely to occur in untreated neurons, more moderate and localized changes of a similar nature are likely taking place during the normal course of neuronal IF metabolism (Okabe et al., 1993).

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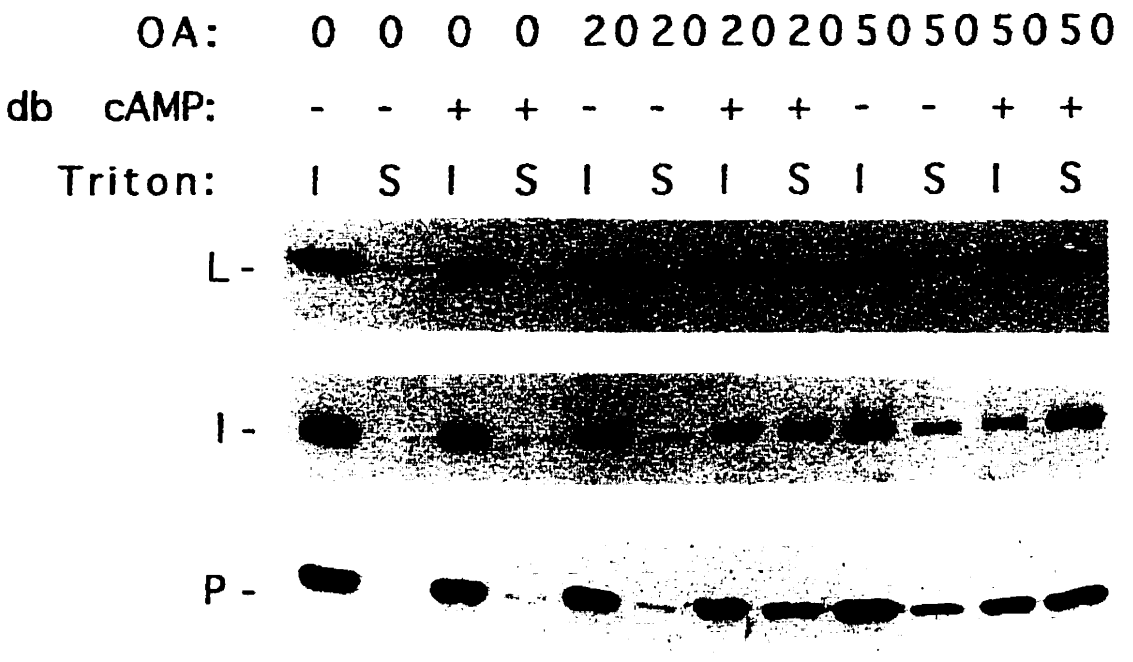
Figures

Figure 1. Western blot analysis of the effect of db cAMP on neuronal IFs in DRG neurons treated with increasing concentrations of OA. DRG cultures were fractionated into Triton X-100-insoluble (I) and -soluble (S) fractions by centifugation at 13,000xg for 30 min. Samples were resolved by SDS-PAGE and the neuronal IF proteins were detected by western blotting as described in Experimental Procedures. The nanomolar OA concentrations are indicated above each lane. Cultures were either pretreated (+) or not treated (-) with 2 mM db cAMP. L, I, and P refer to NFL, α-internexin and peripherin, respectively.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db cAMP: - - + + - - + + - - + +
Triton: I S I S I S I S I S I S
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I -
P -

Figure 2. Phosphorylation of peripherin in DRG cultures treated with db cAMP and OA. DRG cultures were preincubated with $^{32}P_i$ and left untreated (lane 1), treated with 2 mM db cAMP (lane 2) , treated with 50 nM OA (lane 3) or treated with 2 mM db cAMP plus 50 nM OA (lane 4). Peripherin was immunoprecipitated as described in Experimental Procedures. (A) Peripherin was visualized by western blotting. (B) After western blotting, the membrane was rinsed with water, dried and ^{32}P-labelled peripherin was visualized by autoradiography.

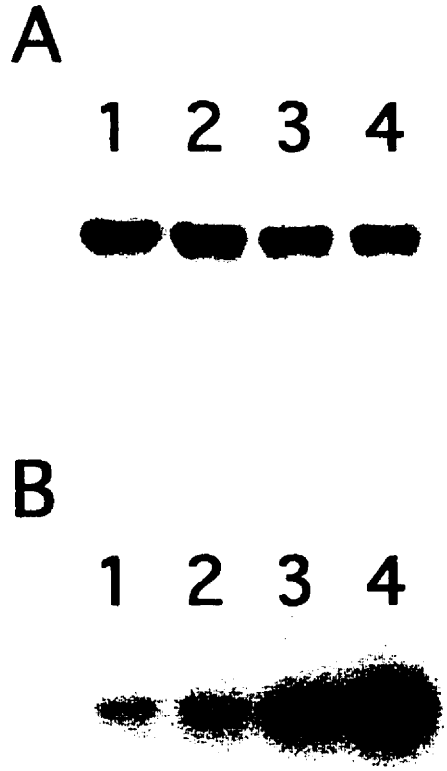
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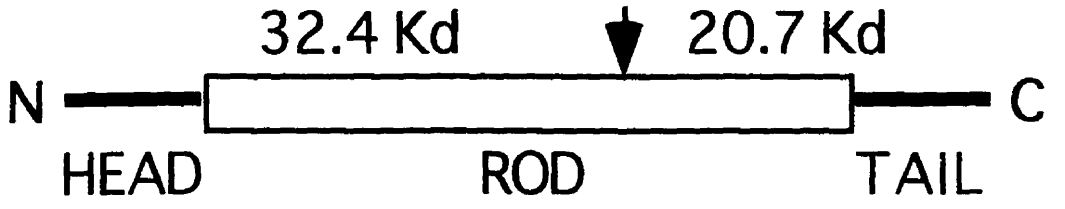
Figure 3. $^{32}P_i$-labelling of the amino- and carboxy-terminal halves of chemically cleaved peripherin from DRG cultures treated with db cAMP and OA. (A) Diagram showing the structure of peripherin. The position of the unique tryptophan residue is indicated by the arrow and the predicted molecular masses of the polypeptides following chemical cleavage at this amino acid residue are indicated above the schematic. (B) DRG cultures were preincubated with $^{32}P_i$ and left untreated (lane 1), or were treated with 2 mM db cAMP (lane 2), 50 nM OA (lane 3) or with 2mM db cAMP plus 50 nM OA (lane 4). Peripherin was immunoprecipitated as described in Experimental Procedures and an equal amount of peripherin protein, as determined by western blotting, was loaded in each lane. The numbers on the right indicate the positions and molecular masses in kDa of protein standards. F, N and C refer to full-length peripherin and the N- and C-terminal segments, respectively.

A



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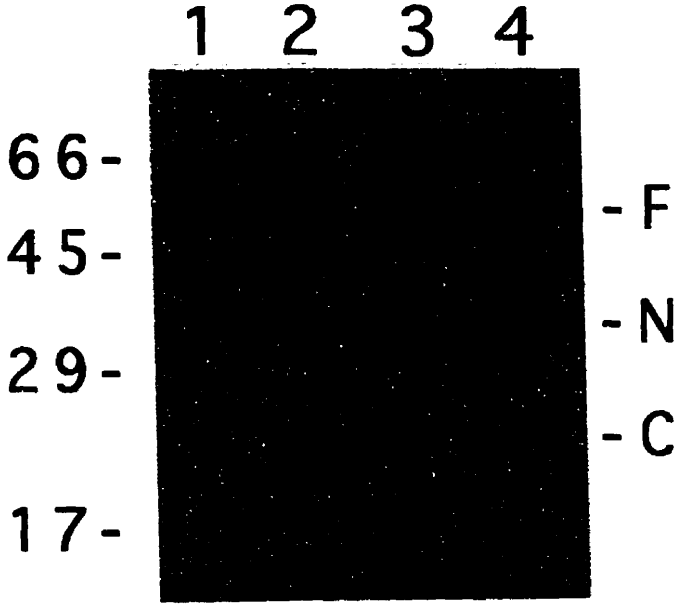

Figure 4. Two-dimensional phosphopeptide mapping of peripherin. DRG cultures were preincubated with $^{32}P_i$ and left untreated (A), or were treated with 2 mM db cAMP (B), 50 nM OA (C) or 2 mM db cAMP plus 50 nM OA (D). Immunoprecipitated peripherin was digested and phosphopeptides were resolved on TLC sheets as described in Experimental Procedures. An equal amount of perpherin, as determined by western blotting, was used for each map. O represents the origin, and the spots numbered 1 to 11 indicate the major peripherin phosphopeptides.

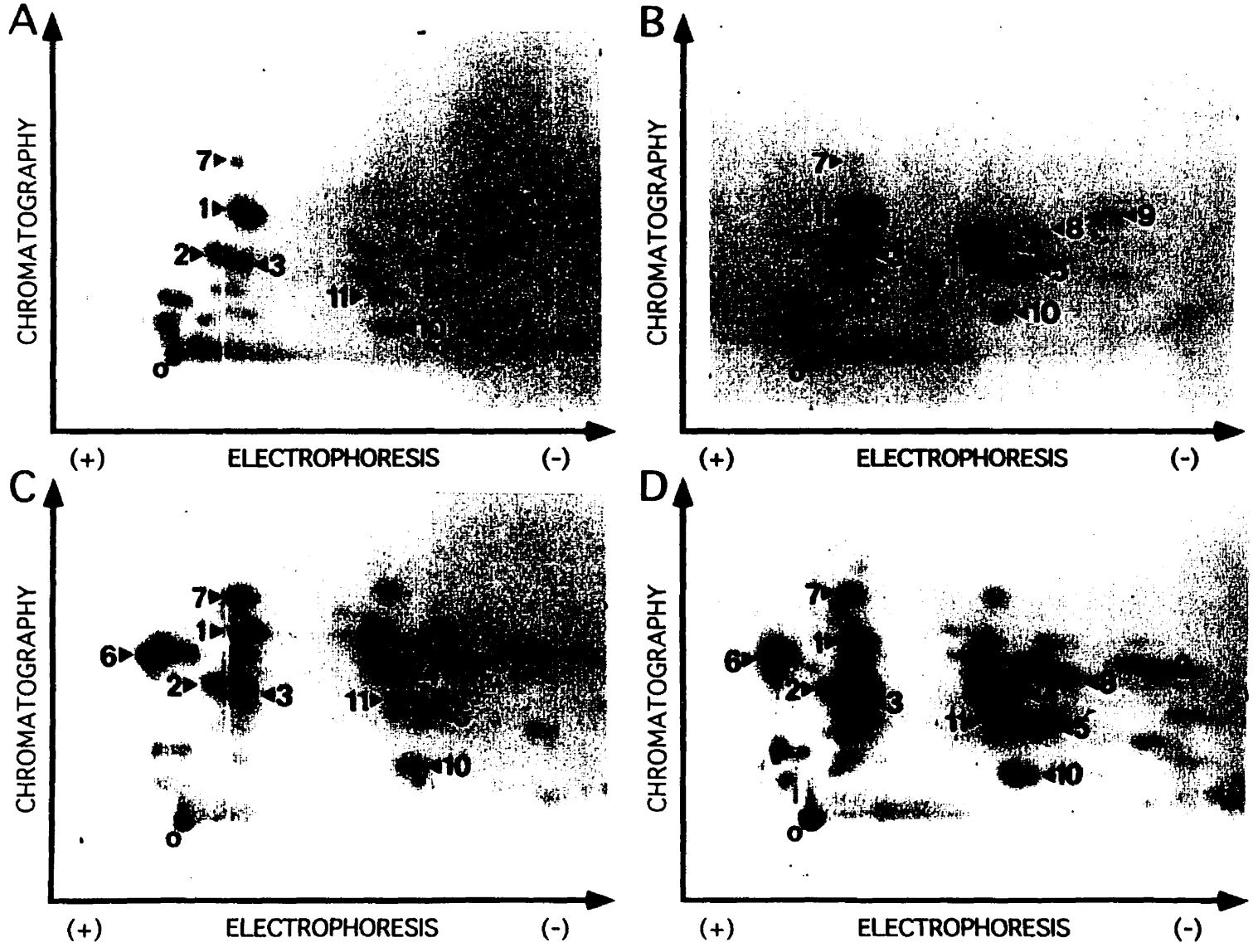
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Figure 5. Two-dimensional phosphopeptide mapping of NFM. **A:** Rat NFM was phosphorylated with PKA in vitro, resolved by SDS-PAGE, and subjected to two dimensional phosphopeptide mapping as described in Experimental Procedures. **B-E:** DRG cultures were preincubated with $^{32}P_i$ and left untreated (B), or were treated with 2 mM db cAMP (C), 50 nM OA (D) or 2mM db cAMP plus 50 nM OA (E). Immunoprecipitated NFM was digested and phosphopeptides were resolved on TLC sheets as described in Experimental Procedures. An equal amount of NFM protein from each DRG culture, based on western blotting, was used for each map. O represents the origin, and the spots labelled with numbers 1 to 9 and asterisks indicate the major NFM phosphopeptides.

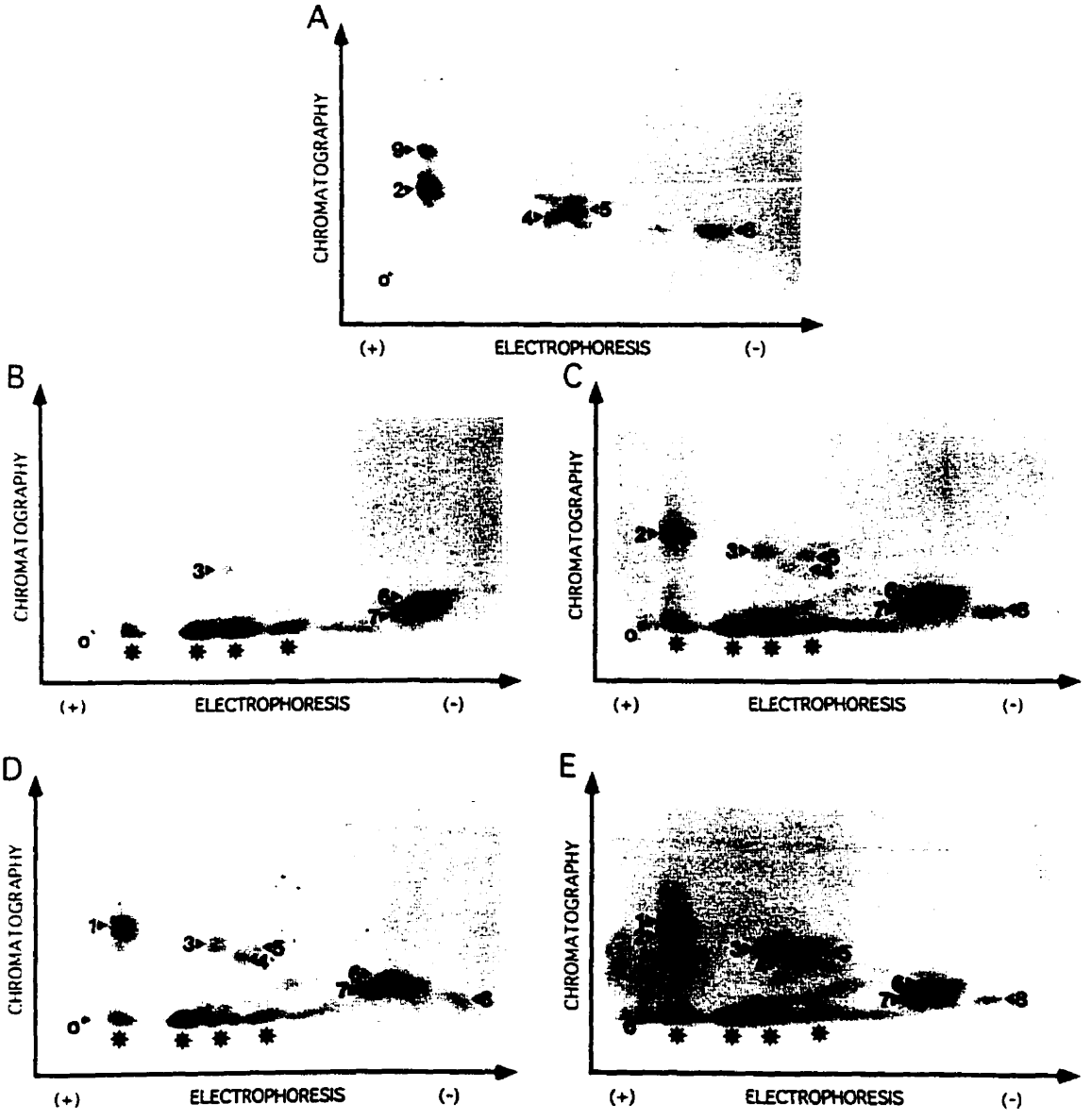
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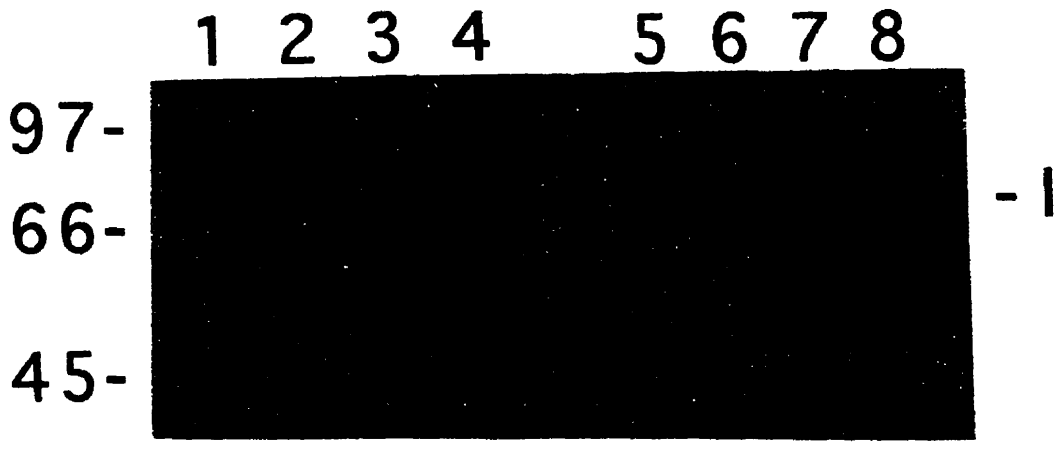
Figure 6. Phosphorylation of α-internexin in DRG and cerebral cortex cultures. DRG cultures (lanes 1-4) and cerebral cortex cultures (lanes 5-8) were preincubated with $^{32}P_i$ and left untreated (lanes 1 and 5), or were treated with 2 mM db cAMP (lanes 2 and 6), 50 nM OA (lanes 3 and 7) or 2 mM db cAMP plus 50 nM OA (lanes 4 and 8). α-internexin was immunoprecipitated as described in Experimental Procedures. (B) α-internexin was visualized by western blotting. (A) After western blotting, the membrane was rinsed with water, dried and ^{32}P-labelled α-internexin was visualized by autoradiography. I refers to α-internexin. The numbers on the right indicate the positions of molecular mass markers and their masses in kDa.

A



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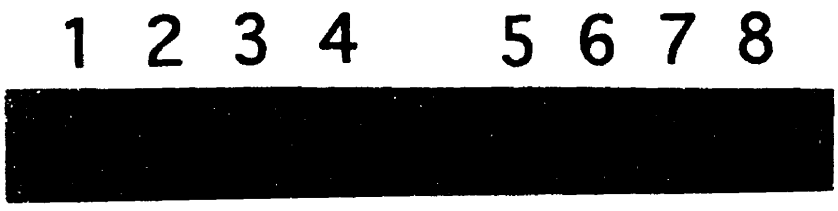

Chapter 4

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NEUROSCIENCE LETTERS

Developmentally regulated stabilization of neuronal intermediate filaments in rat cerebral cortex

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Abstract

The expression and Triton X-100 (Triton) solubility of neuronal intermediate filament proteins were determined in the developing rat cerebral cortex. The level of expression of α-internexin was unchanged from embryonic day 15 (E15) to postnatal day 15 (P15), whereas expression of the mid-sized neurofilament subunit increased continually during this interval concomitant with a reduction in Triton solubility of the two proteins. The low molecular weight neurofilament subunit, first barely detected at P2, was largely insoluble in Triton from the initial time point that its solubility could be assayed, at P5, to P15. Similar expression patterns and Triton solubility profiles were obtained for neuronal intermediate filament proteins in cultured neurons from E15 cerebral cortex. These results suggest that α-internexin is expressed earlier than neurofilament proteins to provide a more plastic network in the early developing brain. The incorporation of neurofilament proteins apparently results in the formation of the more stable intermediate filament network found in mature neurons. © 1997 Elsevier Science Ireland Ltd.

Keywords: α-Internexin; Cerebral cortex; Development; Neurofilament

The 8–10 nm filaments found in most mammalian cells are composed of subunits belonging to the large family of intermediate filament (IF) proteins [7]. The earliest IF proteins expressed in and associated with development of the mammalian nervous system are vimentin [4] and nestin [14]. However, the expression of both proteins is silenced when neuronal precursor cells become postmitotic. Postmitotic neurons may express some or all of five other IF proteins [1,7]. Three of these comprise the neurofilament (NF) triplet, consisting of the low molecular weight (NFL), mid-sized (NFM) and high molecular weight (NFH) NF subunits [9]. α-Internexin (α-Int) and peripherin are the other two IF proteins found in neurons [7]. The expression of α-Int precedes that of other neuronal IF proteins in postmitotic neurons [1,6,11], while peripherin is expressed mainly in the peripheral nervous system [5].

DNA transfection studies with cells deficient in endogenous cytoplasmic IFs demonstrated that NFs are obligate heteropolymers in vivo [3,13]. The co-expression of either NFM or NFH with NFL is required for the formation of an IF network. Unlike NF subunits, α-Int can assemble into an extended filament network in the absence of other IF proteins and can also co-assemble with NF subunits both in vivo [3] and in vitro [2]. This apparent versatility of α-Int in forming homo- and heteropolymeric networks conforms with the suggestion that it forms a transitional IF network that serves as a scaffold for the incorporation of NF subunits [6,15].

This report details our study of how the stabilization of the neuronal IF network is affected by developmental changes in expression of neuronal IF proteins in rat cerebral cortex, as well as in rat cerebral cortex cultures from embryonic day 15 (E15) embryos. A progressive stabilization of the neuronal IF network was observed which coincided with an upregulation in the levels of NF proteins.

Embryos were obtained from Sprague–Dawley rats that were anesthetized with ether and sacrificed by cervical dislocation. Cerebral cortices dissected from E15 rat embryos were dissociated with trypsin and cells were maintained in defined medium N1 containing 30 μg ml^{-1} bovine apo-transferrin, with added 0.9% bovine serum albumin [8], 1% fetal

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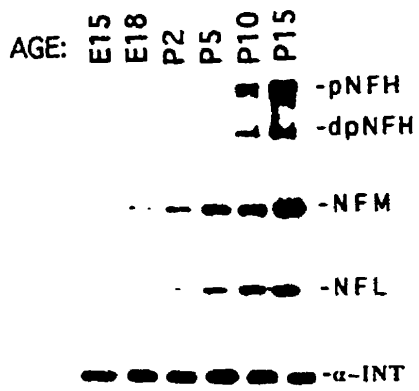

Fig. 1. Expression of neuronal IF proteins in the developing cerebral cortex. The age of the rats at the time of dissection is indicated at the top of each lane. Equal amounts of protein were loaded in each lane and neuronal IF proteins were detected by ECL on Western blots. pNFH and dpNFH refer to hyper- and hypophosphorylated forms of the high molecular weight NF subunit, respectively. NFM, NFL and α-INT refer to the mid-sized and low molecular weight NF subunits and α-internexin, respectively.

calf serum (Gibco BRL) and antibiotics. Culture dishes were coated with crosslinked collagen [8].

For the Triton X-100 (Triton) fractionation of freshly dissected cerebral cortices, the tissue was rinsed in phosphate-buffered saline (PBS) and disrupted in 10 volumes of Triton buffer (1% Triton, 50 mM Tris (pH 7.5), 100 mM NaCl, 2 mM EGTA, 2 mM levamisole, 50 mM NaF and protease inhibitors) using a Dounce homogenizer (10 strokes). The extracts were centrifuged at 13000 × *g* for 20 min. Pellets and supernatants were solubilized in sodium dodecyl sulfate (SDS)-sample buffer [12]. Cerebral cortex cultures were harvested in PBS, lysed in Triton buffer by vortexing and centrifuged as described above.

For experiments requiring the equal loading of proteins for SDS-polyacrylamide gel electrophoresis (PAGE), cerebral cortices were rinsed in PBS and lysed in 2% SDS, 62.5 mM Tris (pH 6.8), and protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce Chemical Co.). Polypeptides were resolved on slab gels by SDS-PAGE [12] and neuronal IF proteins were detected by Western blot analysis [8] using an enhanced chemiluminescence (ECL) detection kit (Amersham). Monoclonal antibodies (anti-NFL, NR4; anti-NFM, NN18; anti-NFH, N52) were obtained from Sigma Chemical Co. Anti-α-Int (MAB1525) and anti-peripherin (MAB1527) monoclonal antibodies were from Chemicon Int. For quantitative Western blotting, [^{125}I]rabbit anti-mouse IgG was used as a secondary antibody and the proteins were quantified using a Fujix BAS 2000 Bio-Imaging Analyzer (Fuji Bio-Imaging).

α-Int was present in the cerebral cortex as a major component as early as E15, and its level of expression remained constant up to postnatal day 15 (P15) (Fig. 1). In contrast, NFM was barely detectable at E15 but its level increased continually up to at least P15, which was the last time point analyzed. NFL was not detectable before P2, and NFH before P5, after which their levels increased up to at least P15. Peripherin was not detected in the cerebral cortex from E15 to P15 (data not shown).

The Triton solubility of NFM and α-Int decreased with increasing age of the rats (Fig. 2). In contrast, most of the NFL was Triton-insoluble from the time (P5) when sufficient amounts were present to assay its solubility. The Triton solubility of NFH could not be quantified by image analysis, because the ^{125}I-based procedure used for quantitative Western blotting is not as sensitive as the ECL detection procedure in Fig. 1 and running higher levels of protein extract on gels caused NFH to smear. However, experiments performed on P10 and P15 extracts using the ECL procedure revealed that the more slowly migrating, hyperphosphorylated isoforms of NFH [10] were predominantly Triton-insoluble, whereas the hypophosphorylated isoforms

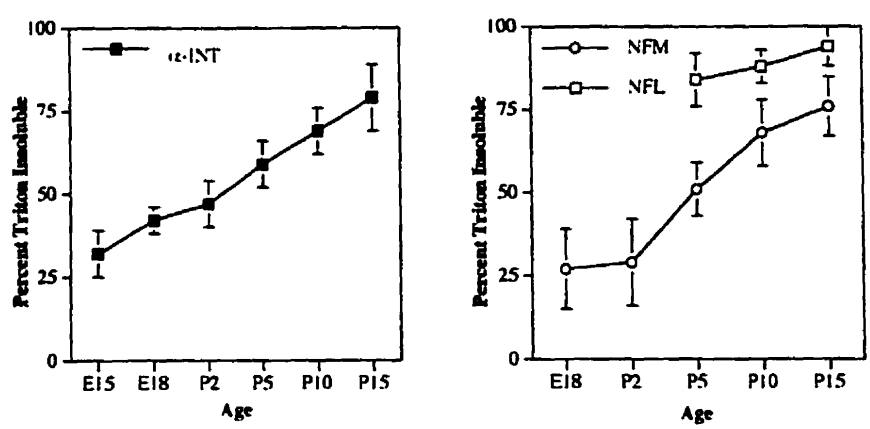

Fig. 2. Changes in Triton solubility of neuronal IF proteins in the developing cerebral cortex. Proteins were separated into Triton-soluble and -insoluble fractions, detected by ^{125}I-based Western blotting and quantified by image analysis. The ages of the rats at the time of the dissections are indicated on the x-axes. NFM, NFL and α-INT are defined in the legend to Fig. 1. $n = 5$–8.

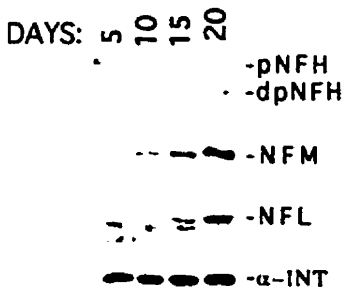

Fig. 3. Expression of neuronal IF proteins in cultures of dissociated E15 rat cerebral cortex. Proteins were detected by ECL on Western blots. The number of days that the cultures were maintained after plating is indicated above each lane. Cultures were harvested in the same volume of SDS-sample buffer and equal volumes of sample were loaded in each lane. IF subunit designations are provided in the legend to Fig. 1.

appeared to be equally distributed between Triton-soluble and -insoluble fractions (data not shown).

In cultures of E15 cerebral cortex, the expression of α-Int was constant throughout the 20 days that neurons were maintained (Fig. 3). NFM could also be detected throughout this interval, although its expression increased with time. NFL was detected only after 15 days and its expression increased from day 15–20, while a band corresponding to hypophosphorylated NFH was first detected at day 10.

The Triton solubility of both NFM and α-Int decreased throughout the time that cerebral cortex neurons were maintained in culture (Fig. 4). Again, NFL was mostly Triton-insoluble from the time it could first be detected. It was not possible to assess the Triton-solubility of NFH in the cultures because its level of expression was too low.

The expression patterns described here for α-Int and the three NF subunits in developing rat cerebral cortex are essentially similar to those reported in previous studies [6,11]. α-Int was by far the predominant neuronal IF protein at E15, the earliest age tested. In addition, the expression of NFM clearly preceded that of NFL both in vivo and in cultured cortical neurons. The early expression of NFM has also been reported for other types of neurons [17,19], contrary to the notion that the expression of NFL and NFM are always tightly linked [15,16,18].

The functional significance of the sequential appearance of different IF proteins in neurons is unknown. It has been suggested that the expression of α-Int prior to the other neuronal IF proteins in postmitotic neurons may be of help to maintain plasticity during neurite outgrowth [15] and to provide a scaffold for subsequent incorporation of NF subunits [6,15]. We show in the present study that the latter event coincides with a decrease in the Triton solubility of neuronal IFs.

At early developmental stages (E15), when it was the major IF protein expressed in cortical neurons, α-Int was largely Triton-soluble. The relative increase in NF subunit levels with continued development was accompanied by a stabilization of the neuronal IF network, as judged by the decrease in Triton solubility of the various components (Figs. 2 and 4). It is interesting to note that NFL was largely insoluble in Triton whenever there were sufficient amounts to be assayed, indicating that it was rapidly incorporated into a stable IF network.

Our proposal that stabilization of neuronal IFs in developing cerebral cortex is due to the incorporation of increasing levels of NF subunits into a network formed initially by α-Int is based on two assumptions: that α-Int and the NF triplet are co-expressed in the same neurons and that they can co-polymerize. There is a great deal of evidence suggesting that both of these assumptions are in fact correct. α-Int is found in most, if not all, central nervous system (CNS) neurons. Its expression precedes that of NF triplet proteins in the embryo and its distribution in the adult CNS closely parallels that of the NF subunits [6,11]. Small cerebellar granule cells appear to represent one of the rare exceptions to the co-localization of these neuronal IF proteins, as they contain only α-Int [6]. There is also considerable evidence indicating that α-Int and NF subunits can co-polymerize both in vivo [3] and in vitro [2]. Also noteworthy is the observed co-immunoprecipitation of α-Int with NF subunits from extracts of okadaic acid-treated sensory neurons, suggesting that these proteins form a highly integrated structure [1].

α-Int is unique among the type IV IF proteins in that it can also form a homopolymeric filamentous network in transfected, IF-deficient cells although about half of the α-Int is Triton-soluble at 13 000 × *g* under these circumstances [3]. This ability to assemble into homo- and heteropolymeric IFs would enable α-Int to form a more plastic, transient IF network in developing neurons and to participate with the NF subunits in forming more stable IFs in mature CNS neurons [15].

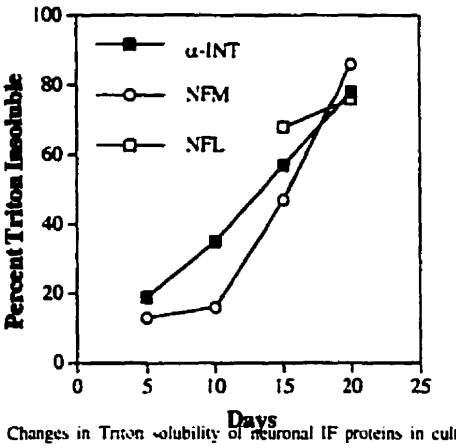

Fig. 4. Changes in Triton solubility of neuronal IF proteins in cultured neurons from dissociated E15 cerebral cortex. The percentage of each IF protein that is Triton-insoluble was determined as described in Fig. 2. The numbers of days that the cultures were maintained after plating are indicated on the *x*-axis. NFM, NFL and α-INT are defined in the legend to Fig. 1.

It is not clear at present whether the effect of NF triplet proteins on the stability of the IF network in cortical neurons is direct or indirect. Stabilization may be the direct result of co-polymerization of NF subunits with α-Int. Alternatively, NF subunits integrated into the IF network may stabilize it by interacting with other cellular components. Resolving this issue will provide new insights into the dynamics of neuronal IFs and their role in neuronal development.

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Chapter 5

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Aberrant Stress-induced Phosphorylation of Perikaryal Neurofilaments*

(Received for publication, June 7, 1996, and in revised form, August 15, 1996)

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The aberrant phosphorylation of the neurofilament high molecular weight subunit (NFH) in the neuronal perikaryon is a common feature of several neurological diseases. We demonstrated a strong correlation between hyperphosphorylation of the NFH carboxyl-terminal domain and activation of stress-activated protein kinase (SAPK) -γ in PC12 cells. Agents that activated SAPKγ in PC12 cells also caused the hyperphosphorylation of perikaryal NFH in cultured dorsal root ganglion neurons. The NFH carboxyl-terminal domain was phosphorylated by SAPKγ *in vitro*, and the use of peptide substrates indicated that this event occurred preferentially at KSPXE motifs. We propose that SAPKγ, perhaps in concert with other SAPKs, is involved in the abnormal phosphorylation of perikaryal NFH. This finding could lead to new insights into the etiology of several neurological diseases.

Neurofilaments (NFs)[1] are major components of the cytoskeleton in many types of mature neurons and are particularly abundant in large, myelinated axons (1). Mammalian NFs are obligate heteropolymers (2–4) composed of three type IV intermediate filament proteins, NFL, NFM, and NFH, with apparent molecular masses of 68, 150, and 200 kDa, respectively (5). In common with other intermediate filament proteins, each NF subunit contains a highly conserved α-helical rod domain, involved in coiled-coil dimer formation, flanked by an amino-terminal head domain and a carboxyl-terminal tail domain (6). The size differences between NF subunits are due to variations in the length of the tail domains (6), which project laterally from the filament axis (7, 8) and appear to form cross-bridges between NFs and with other axonal structures (9).

The tail domains of NFM and NFH display complex subdomain patterns with several distinct motifs (10). One such motif, the Lys-Ser-Pro (KSP) sequence, is especially abundant in the tail domain of NFH, with numbers ranging from 43 in human NFH (11) to over 50 in NFH from mouse (12) and rat (13). These KSP repeats represent the major phosphorylation sites in NFH and NFM (14, 15), which account for the high phosphoserine content of the two proteins (7, 16). The electrophoretic mobilities of NFH and NFM on SDS-polyacrylamide gels are retarded significantly by phosphorylation of the KSP repeats (16).

The knowledge that KSP repeats contain the major phosphorylation sites in NFM and NFH has focused efforts to identify the relevant kinases within the superfamily of proline-directed protein kinases. Several neuronal enzymes in this category have been shown to phosphorylate NFH *in vitro*. These include glycogen synthase kinase-3 (17), extracellular signal-regulated kinases (ERKs) (18, 19), and cyclin-dependent kinase-5 (20, 21). Since KSP motifs located in different sequence contexts are phosphorylated *in vivo* (15), several kinases may participate in tail domain phosphorylation, perhaps through a hierarchical mechanism (22).

The development of monoclonal antibodies that could distinguish between phosphorylated and unphosphorylated KSP repeats in the tail domains of NFH and NFM led to the discovery that axonal NFs are normally more highly phosphorylated than those located in the perikaryon and dendrites (23, 24). This normal phosphorylation pattern is characteristically altered in a variety of neuropathologies, where perikaryal NFs become hyperphosphorylated (25, 26).

We recently showed (27) that *N*-acetyl-Leu-Leu-norleucinal (CI), a potent calpain (28) and proteasome inhibitor (29, 30), stimulated phosphorylation of the tail domain of NFH in neuron-like PC12 cells and in cell bodies of dorsal root ganglion (DRG) neurons. We have now found that agents known to activate stress response pathways have an effect similar to that of CI. These pathways involve proline-directed kinases of the mitogen-activated protein (MAP) kinase family, which include the ERKs, the stress-activated protein kinases (SAPKs), and p38. (31). The MAP kinases are related structurally and are activated by similar cascades in response to diverse external stimuli. The SAPK pathway responds to intra- and extracellular stress stimuli and may promote inhibition of cell growth (32, 33). There is a degree of cross-talk between the different MAP kinase signaling cascades as evidenced by the activation of ERKs, SAPKs, and p38 by hyperosmolarity (34, 35). However, the various MAP kinases also respond differently to certain stimuli, one example being the activation by arsenite of p38, but not the ERKs, in PC12 cells (36).

We report here that CI caused the prolonged activation of ERK-1/2 and SAPKγ. The stimulation of NFH tail domain phosphorylation in PC12 cells by various stress response activators correlated with the degree of SAPKγ activation and the kinase phosphorylated recombinant NFH tail domain *in vitro*. These results suggest that stress-activated protein kinases may be responsible for the hyperphosphorylation of perikaryal NFs seen in various neuropathologies.

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‡ Recipient of a Studentship from the Fonds de la Recherche en Santé du Québec.

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[1] The abbreviations used are: NF, neurofilament; CI, *N*-acetyl-Leu-Leu-norleucinal; CII, *N*-acetyl-Leu-Leu-methioninal; DRG, dorsal root ganglia; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; NFL, NFM, and NFH, low, medium, and high molecular mass neurofilament subunits, respectively; NGF, nerve growth factor; KSP, Lys-Ser-Pro; PAGE, polyacrylamide gel electrophoresis; SAPK, stress-activated protein kinase; TNFα, tumor necrosis factor-α; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

EXPERIMENTAL PROCEDURES

Materials—CI, *N*-acetyl-Leu-Leu-methioninal (CII), anisomycin, and anti-NFH monoclonal antibody N52 were purchased from Sigma. Human recombinant tumor necrosis factor-α (TNFα) was from Collaborative Biomedical Products (Bedford, MA). Nerve growth factor (NGF) (2.5S) was purchased from Prince Laboratories (Toronto, ON). Anti-NF monoclonal antibodies SMI 31 and SMI 34 were obtained from Sternberger Monoclonals, Inc. (Baltimore, MD). Anti-SAPKγ (C-17), anti-ERK-1 (C-16), and anti-ERK-2 (C-14) polyclonal antibodies as well as glutathione *S*-transferase (GST)-c-Jun (amino acids 1–79) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Myelin basic protein and bovine serum albumin were from Life Technologies, Inc. (Burlington, ON). Bovine apotransferrin was from ICN (Mississauga, ON). GST-NFH fusion protein containing the entire tail domain of murine NFH (amino acids 412-1087; Ref. 12) was expressed as a recombinant protein in *Escherichia coli* using the bacterial expression vector pGEX-2T (Pharmacia Biotech, Baie D'Urfé, PQ) and was purified by affinity chromatography using glutathione-Agarose.

Cell Culture—Embryos were obtained from Sprague-Dawley rats that were previously anesthetized with ether and sacrificed by cervical dislocation. DRGs from E15 rat embryos were dissected, dissociated with trypsin, and maintained in defined medium N1 (37) containing 30 μg ml^{-1} bovine apotransferrin, with added 0.9% bovine serum albumin, 6 ng ml^{-1} 2.5 S NGF, and antibiotics. Culture dishes were coated with cross-linked collagen (38) in a procedure involving overnight precipitation of 50 μg ml^{-1} collagen followed by cross-linking for 2 h at room temperature with 130 μg ml^{-1} of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-*p*-toluenesulfonate. Dissociated DRGs were plated either dispersely or in a volume of 10 μl at the center of a 35-mm dish. In the latter case, the cells were allowed to attach for 30 min before flooding with defined medium. The localization of neuronal perikarya in a small centrally located region allowed them to be separated manually from the surrounding halo of neurites. Localized and dispersed DRG cultures were maintained for 19–20 days before being used for experiments.

The PC12 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained according to published procedures (39, 40).

PC12 cells were grown in 85% Dulbecco's modified Eagle's medium (high glucose), 10% heat-inactivated horse serum, 5% fetal bovine serum (Life Technologies, Inc.), and antibiotics.

Immunoprecipitation Kinase Assay of SAPKγ—The cells were harvested in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and lysed with 200 μl of SAPKγ lysis buffer (20 mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} leupeptin). Cell debris was removed by centrifugation at 13,000 × *g* for 5 min at 4 °C. An aliquot of each supernatant was used to determine the protein concentration and equalize the amount of protein used in each set of experiments. SAPKγ was immunoprecipitated from 100 to 150 μg of total protein using 10 μl of anti-SAPKγ polyclonal antibody (C-17) (100 μg ml^{-1}) and 20 μl of protein A-Sepharose CL-4B suspension (Pharmacia Biotech, Baie D'Urfé, PQ) for 3 h at 4 °C. The immunocomplexes were washed four times with SAPKγ lysis buffer and once with SAPKγ kinase buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate). The assays were initiated by adding to the sedimented beads 10 μl of SAPKγ kinase buffer containing 0.5 μg of GST-c-Jun and 50 μM [γ-^{32}P]ATP (5 Ci/mmol). The reactions were incubated at 30 °C for 20 min and then terminated by boiling for 5 min in SDS sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol). Phosphorylation of the substrate protein was visualized after SDS-polyacrylamide gel electrophoresis (PAGE) (41) by autoradiography and quantified using a Fujix BAS2000 Bio-Imaging Analyzer (Fuji Bio-Imaging).

Immunoprecipitation Kinase Assay of ERK-1/ERK-2—The assay was similar to that for SAPKγ except for the following changes. The lysis buffer consisted of 50 mM NaCl, 5 mM EGTA, 10 mM Tris, pH 7.6, 0.2% Nonidet-P40, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 10 μg/ml leupeptin. The kinases were immunoprecipitated with 5 μl each of anti-ERK-1 (C-16) (100 μg/ml) and anti-ERK-2 (C-14) (100 μg/ml) polyclonal antibodies. ERK kinase buffer consisted of 30 mM Hepes, pH 7.2, 10 mM $MgCl_2$, and 1 mM dithiothreitol. Assays were initiated by adding to the sedimented beads 20 μl of ERK kinase buffer containing 10 μg of myelin basic protein and 50 μM [γ-^{32}P]ATP (5 Ci/mmol). The reactions were terminated after 20 min at 30 °C by boiling for 5 min in SDS sample buffer. The phosphorylation of myelin basic protein was quantified by liquid scintillation counting of Coomassie Blue R250-staining protein bands excised from SDS-PAGE gels.

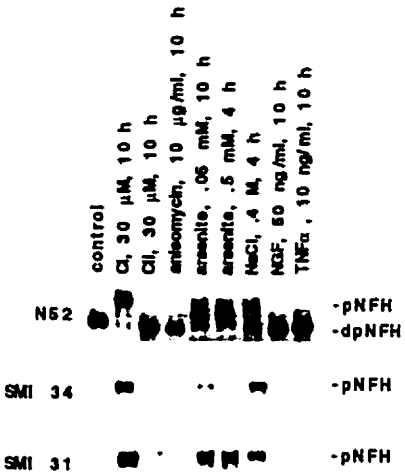

FIG. 1. **NFH carboxyl-terminal tail domain phosphorylation in PC12 cells.** Western blot analysis of protein extracts from PC12 cells treated with various agents and probed with anti-NFH antibodies N52, SMI 31, and SMI 34. The agent and duration of treatment are indicated *above* each lane. *pNFH* and *dpNFH* refer to hyper- and hypophosphorylated NFH, respectively. Equal amounts of protein were loaded in each lane.

Gel Electrophoresis and Western Blotting—For the separate analysis of neurite and perikaryal fractions, dissociated DRGs were plated on a small area at the center of a dish as described above. This allowed for the manual separation of a central zone rich in neuronal cell bodies from the halo of neurites by using a circular punch with a diameter slightly larger than that of the neuronal cell body mass. The DRG fractions were solubilized in SDS sample buffer. PC12 cells were harvested in 2% SDS, 50 mM Tris, pH 6.8, and protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce). Equal amounts of protein were resolved on slab gels by SDS-PAGE (41). Proteins were electrophoretically transferred to Immobilon-P membrane (Millipore Corp.) in buffer containing 48 mM Tris, 39 mM glycine, and 5% methanol. The membrane was then blocked with 1% skimmed milk powder in Tris-buffered saline/Tween (20 mM Tris, pH 7.7, 137 mM NaCl, and 0.1% Tween 20), incubated with primary antibodies, and developed using the ECL Western blotting Detection Kit (Amersham Corp.).

Immunofluorescence Microscopy—Dispersed DRG cultures were grown on collagen-coated glass coverslips. The cells were fixed with 3.7% phosphate-buffered formaldehyde, then with methanol at −20 °C, rinsed, blocked with 50% goat serum in TBS (10 mM Tris, pH 7.4, 150 mM NaCl), 0.3% Triton X-100, and incubated for 30 min with SMI 34 monoclonal antibody (1/1000) in TBS, 0.3% Triton X-100 containing 10% goat serum. After extensive washing, the cells were incubated with Texas Red-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in TBS, 0.3% Triton X-100 containing 10% goat serum.

RESULTS

Effect of Different Agents on NFH Phosphorylation in PC12 Cells—The effects on NFH phosphorylation of agents that are known to activate members of the MAP kinase family were assessed by Western blotting using three different monoclonal antibodies. N52 is a NFH-specific, phosphorylation-independent antibody (42) that was used to assess increases in NFH phosphorylation by detecting species with a reduced mobility on SDS-PAGE (16). SMI 31 and SMI 34 antibodies bind to different phosphoepitopes involving KSP repeats in the tail domains of NFH and sometimes NFM (43).

Treatment of PC12 cells with CI (30 μM) for 10 h caused all

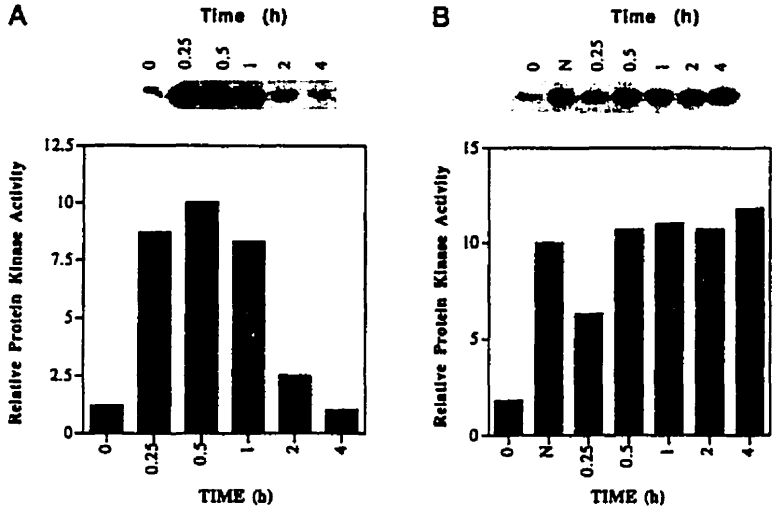

FIG. 2. **Activation of SAPKγ in PC12 cells.** Time course of SAPKγ activation in PC12 cells treated with 0.4 M NaCl (*A*) or 0.5 mM sodium arsenite (*B*). The lane in *B* labeled with an *N* represents an internal control in which the cells had been treated with 0.4 M NaCl for 30 min. Immunoprecipitation kinase assays with GST-c-Jun as substrate were performed as described under "Experimental Procedures." Similar results were obtained in two independent experiments.

of the NFH to shift to a more slowly migrating form (Fig. 1) and resulted in the greatest increase in NFH immunoreactivity to SMI 31 and SMI 34 among the various agents that were tested. Treatment of PC12 cells with CII (30 μM) for 10 h did not cause any detectable change in NFH phosphorylation. NGF, an activator of ERKs in PC12 cells (44, 45), also had no noticeable effect on NFH phosphorylation (Fig. 1). Neither did treatment with anisomycin or TNFα, two SAPK activators (46–48).

Arsenite and osmotic shock, which have been shown to activate stress response pathways in PC12 cells (36, 49), each caused a partial shift in the mobility of NFH (Fig. 1). Increased osmolarity rendered NFH immunoreactive with both SMI 31 and SMI 34, whereas arsenite treatment produced NFH that was more immunoreactive with SMI 31. PC12 cells were treated with 0.4 M NaCl or 0.5 mM arsenite for no longer than 4 h, as they began detaching from the substratum beyond this time point. Treatment with 0.05 mM arsenite for 10 h yielded results similar to those with 0.5 mM arsenite for 4 h. The band slightly above NFH that was weakly reactive with SMI 31 antibody did not appear to be a slower migrating form of NFH, since it was not detected by N52 antibody.

SAPKγ Activation Parallels the Increase in NFH Phosphorylation—Since only certain of the agents known to activate SAPKs caused an increase in NFH phosphorylation, we determined whether the discrepancies were due to variation in the extent of enzyme activation. Hyperosmolarity produced a relatively strong activation of SAPKγ in PC12 cells, and the high level of activity was maintained for approximately 1 h (Fig. 2*A*). Arsenite produced approximately the same level of activation as hyperosmolarity, although the activation was more prolonged (Fig. 2*B*). The increase in NFH phosphorylation was nevertheless comparable in the two cases (Fig. 1), suggesting that toxicity may have dampened the effect of more prolonged activation by arsenite. Anisomycin produced a very modest level of SAPKγ activation which peaked at 0.5 h (Fig. 3*A*). TNFα also caused only a modest and transient activation of SAPKγ (Fig. 3*B*), similar to that reported in HeLa cells (48). CI produced a very strong and prolonged activation of SAPKγ which lasted for at least 10 h (Fig. 3*C*). CII also caused a prolonged activation of SAPKγ but at a lower level than obtained with CI (Fig. 3*D*).

Activation of ERK-1/2 by NGF, CI, and CII—Since ERK-2 has been implicated in the phosphorylation of NFH *in vitro* (18, 19), we compared the effects of CI and CII on ERK-1/2 activity

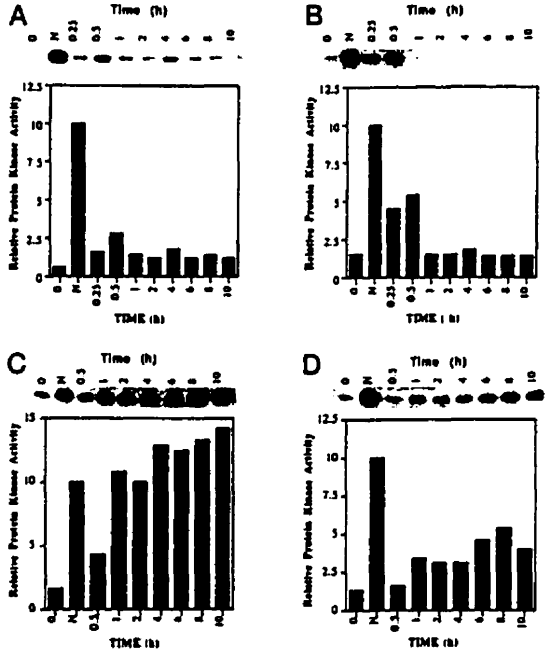

FIG. 3. **Activation of SAPKγ in PC12 cells.** Time course of SAPKγ activation in PC12 cells. Cells were treated with either 10 μg ml^{-1} anisomycin (*A*), 10 ng ml^{-1} human, recombinant TNFα (*B*), 30 μM CI (*C*), or 30 μM CII (*D*). Other details are the same as in the legend to Fig. 2. Similar results were obtained in two independent experiments.

with that of NGF, a known ERK activator. NGF caused a prolonged activation of ERK-1/2 (Fig. 4) similar to what has already been reported (44, 45). Prolonged ERK activation was also seen following CI treatment, but at a lower level than was obtained with NGF. The lowest level of ERK activation was observed with CII treatment.

Increased Phosphorylation of Perikaryal NFH in DRG Neurons—As shown in Fig. 5, CI treatment of DRG cultures produced a complete shift in the mobility of perikaryal NFH, to a level normally seen only in axonal NFH. CII, which did not cause an observable increase in NFH phosphorylation in PC12

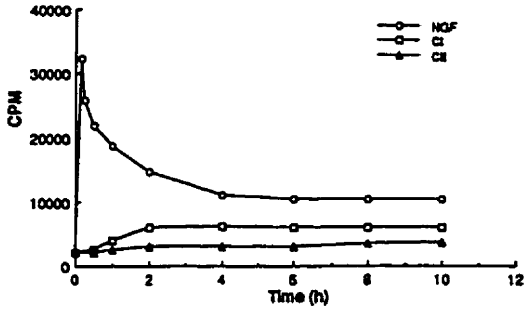

FIG. 4. **Activation of ERK-1/2 in PC12 cells.** Time course of ERK-1/2 activity in PC12 cells treated with CI (30 μM), CII (30 μM), or 2.5 S NGF (50 ng ml^{-1}). Immunoprecipitation kinase assays with myelin basic protein as substrate were performed as described under "Experimental Procedures." Similar results were obtained in three independent experiments.

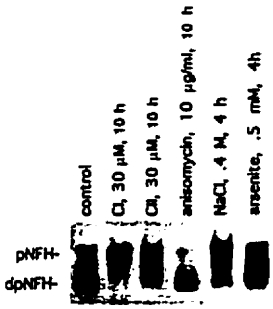

FIG. 5. **Perikaryal NFH phosphorylation in DRG neurons.** Western blot analysis of NFH from neuronal perikarya of DRG cultures. Localized DRG cultures were prepared as described under "Experimental Procedures." The cultures were maintained for 19–20 days and then treated as indicated *above* each lane. The neuronal cell bodies were manually separated from the neurites as described under "Experimental Procedures" and subjected to Western blot analysis using the anti-NFH monoclonal antibody, N52. *pNFH* and *dpNFH* refer to hyper- and hypophosphorylated NFH, respectively.

cells, effected a partial decrease in the mobility of perikaryal NFH in DRGs. Hyperosmotic shock caused a more extensive shift in the mobility of perikaryal NFH. Arsenite treatment caused a broadening of the NFH band, whereas anisomycin treatment had no effect.

CI treatment and hyperosmotic shock promoted the staining of neuronal perikarya with SMI 34 (Fig. 6, *B* and *D*, respectively). Treatment with CII (Fig. 6*C*), anisomycin, or arsenite (data not shown) did not enhance the staining of neuronal perikarya with SMI 34.

Since hyperphosphorylation of perikaryal NFH and NFM has been observed following axonal damage (50), we tested whether this effect could be reproduced *in vitro*. The neurites of DRG neurons in localized cultures were severed from their cell bodies, and the latter were harvested at different times after injury. The mobility of NFH on SDS-PAGE was unchanged at 2 and 5 h following neurite disruption (Fig. 7). A partial decrease in mobility was seen at 12 and 24 h, which was reversed by 48 h. It is likely that the elevated phosphorylation state of perikaryal NFH that occurs in both mechanically damaged cultures and in cultures exposed to other stressing agents is due to the activation of the same or related kinase(s).

Phosphorylation of NFH and Peptides by SAPKγ—GST-NFH is a recombinant fusion protein that contains the entire tail domain of mouse NFH (amino acids 412-1087; Ref. 12) in a

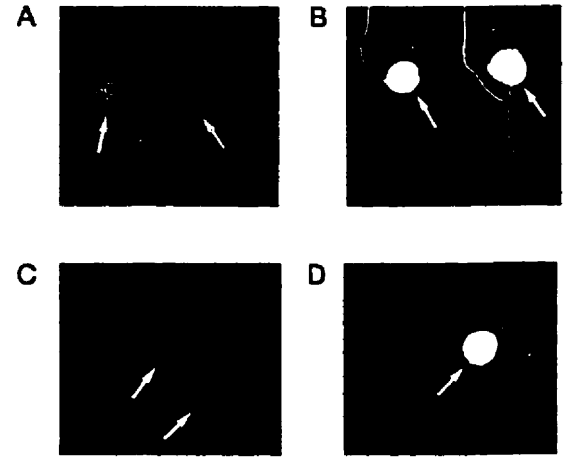

FIG. 6. **Anti-SMI 34 immunoreactivity of DRG neuronal cell bodies.** Immunofluorescence micrographs of control DRG cultures (*A*) or cultures treated for 10 h with 30 μM CI (*B*), 10 h with 30 μM CII (*C*), or 4 h with 0.4 M NaCl (*D*). The cultures were stained with SMI 34 antibody. *Arrows* point to neuronal perikarya.

FIG. 7. **Western blot analysis of perikaryal NFH in DRG cultures after mechanical damage.** The neurites were manually severed from the cell bodies using a punch with a diameter slightly larger that the cell body mass, and the cell bodies were harvested at different times indicated *above* each lane. The samples were subjected to Western blot analysis using the anti-NFH monoclonal antibody, N52. *pNFH* and *dpNFH* refer to hyper- and hypophosphorylated NFH, respectively.

completely unphosphorylated state.[2] GST-NFH was phosphorylated by SAPKγ in an immunoprecipitation kinase assay (Fig. 8*A*), whereas GST alone was not phosphorylated under the same conditions (data not shown). GST-NFH was not as good a SAPKγ substrate as GST-c-Jun; 30–35 times more ^{32}P was incorporated into GST-c-Jun than GST-NFH when the immunoprecipitation kinase assays were done in parallel.

The KSP repeats in NFH contain different motifs. Three of the most abundant motifs are KSP*X*E, KSPEK, and KSP*X*K, where *X* is an uncharged amino acid residue, usually an Ala, a Gly, or a Val. Peptide-(601–615) (EAKSPAEAKSPAEAK) and peptide-(854–867) (VKSPAKEKAKSPEK) correspond to the amino acid sequence in murine NFH (12). Peptide-(601–615) contains 2 KSP*X*E motifs, and peptide-(854–867) contains both KSP*X*K and KSPEK motifs. Immunoprecipitation kinase assays of SAPKγ demonstrated that peptide-(601–615) was phosphorylated at a markedly higher level than peptide-(854–867) (Fig. 8*B*).

DISCUSSION

In this study we have shown that treatment of PC12 cells and DRG neurons with agents that activate stress response pathways can promote the phosphorylation of KSP repeats in the tail domain of NFH. The extent to which NFH phosphorylation in PC12 cells was increased correlated with the degree of SAPKγ activation by various agents. The strongest activator, CI, as well as hyperosmotic shock, also increased the phosphorylation of perikaryal NFH in DRG neurons. Although other stress-activated kinases, in addition to SAPKγ, may also participate in this process, the ERKs did not appear to play a

[2] M. G. Sacher, unpublished results.

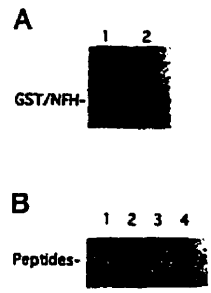

FIG. 8. **Phosphorylation of NFH and substrate peptides by SAPKγ.** *A*, PC12 cells were either not treated (*lane 1*) or treated (*lane 2*) with 0.4 M NaCl for 30 min. Immunoprecipitation kinase assays of SAPKγ using GST-NFH as substrate were performed as described under "Experimental Procedures." *B*, PC12 cells were either not treated (*lanes 1* and *3*) or treated with 0.4 M NaCl for 30 min. Immunoprecipitation kinase assays of SAPKγ were performed as described under "Experimental Procedures." Peptide-(601–615) (EAKSPAEAKSPA-EAK) (*lanes 1* and *2*) and peptide-(854–867) (VKSPAKEKAKSPEK) (*lanes 3* and *4*) at a concentration of 0.4 mM were used as substrates in the kinase assay. The peptides were resolved on a 16.5% acrylamide, 6% bisacrylamide gel using Tricine-polyacrylamide gel electrophoresis (77) and were visualized by autoradiography.

significant role in view of the failure of NGF treatment to cause a detectable increase in the phosphorylation state of NFH (Figs. 1 and 4). In addition, treatment of PC12 cells with arsenite, which activates SAPKγ (Fig. 2), but not ERKs (36), caused an increase in NFH phosphorylation.

The evidence linking SAPKγ to the hyperphosphorylation of NFH is compelling. There was a strong correlation between the extent of KSP repeat phosphorylation and the degree of SAPKγ activation in PC12 cells. Only when SAPKγ was strongly activated, as in the case of treatment with 30 μM CI, 0.4 M NaCl, or 0.5 mM arsenite, was there a detectable increase in phosphorylation of the tail domain of NFH. More modest increases in SAPKγ activity caused by TNFα, anisomycin, or 30 μM CII were without apparent effect. However, lower levels of phosphorylation in the latter cases could have gone undetected since multiple phosphorylation events are required to cause the extensive shifts in gel electrophoretic mobility (16), and concurrent appearance of phosphoepitopes (14), that were monitored in the present study.

In addition to these suggestive correlations, we have shown that SAPKγ phosphorylates recombinant NFH tail domain *in vitro*, as well as a peptide with KSPXE sequence motifs that occur in NFH. Recombinant NFH was not as good an *in vitro* substrate for SAPKγ as c-Jun. This could explain the need for strong activation of SAPKγ in PC12 cells to obtain detectable shifts in NFH mobility and the concurrent appearance of phosphoepitopes. The SAPK phosphorylation sites in the amino-terminal domain of c-Jun consist of a Ser followed by a Pro and an acidic residue (SPD or SPE) (51). Similar although not identical sequences (SPXE) in the tail domain of murine and rat NFH are relatively frequent (12, 13), and SAPKγ phosphorylated the Ser in these sequences in peptide-(601–615) (Fig. 8*B*). Peptide-(854–867) was a poorer SAPKγ substrate; perhaps the Lys residue that immediately follows Glu in the KSPEK motif has a neutralizing effect that renders the site an unsuitable substrate.

The widespread distribution of SAPKs and ERKs in the nervous system (52) provides further support for our proposal that the hyperphosphorylation of perikaryal NFs is due to the activation of stress response pathways. Other members of the MAP kinase family in addition to SAPKγ may be similarly involved. Differential responses to a given stressing agent might occur in different types of neurons, depending on the prominence of appropriate sensing mechanisms and on relative levels of the various MAP kinases. This may be why CII stimulated NFH phosphorylation in DRG neurons but not in PC12 cells. CII also inhibits calpains and proteasomes, although the IC_{50} value is approximately 5–10-fold higher than for CI (28, 30). If MAP kinases other than SAPKγ are also involved in the neuronal stress response, they would augment the action of SAPKγ, the end result being aberrant NF phosphorylation.

ERKs and SAPKs have also been implicated in the neuronal differentiation of PC12 cells (44, 53). The activation by CI of both ERK-1/2 and SAPKγ (Figs. 3*C* and 4) could explain its ability to induce neurite outgrowth in PC12 cells (54).

The finding that stress-activated kinases can phosphorylate perikaryal NFs has obvious clinical implications. Abnormal phosphorylation and accumulation of perikaryal NFs occur together in several neuropathologies, suggesting that the two are somehow linked (25, 26). These two characteristics are seen in neurodegenerative diseases such as Alzheimer's (55), Parkinson's (56), and amyotrophic lateral sclerosis (57–59). It is possible that a stress response activator, such as oxidative stress (60), causes the premature phosphorylation of perikaryal NFs leading to their accumulation. Other forms of stress could produce similar effects, which would be consistent with the multifactorial nature of amyotrophic lateral sclerosis (61). NF accumulations in the perikaryon or proximal axon of motor neurons have been shown to block axonal transport of NFs, tubulin, actin, and mitochondria and could eventually cause axonal degeneration (62).

NF subunits are synthesized in the perikaryon and move down the axon in the slow axonal transport compartment (5) until they reach the nerve terminal, where they are degraded (63). Phosphorylation of the KSP repeat domains in NFM and NFH normally commences in the initial axon segment and continues during transport (64–66). The notion that aberrant tail domain phosphorylation may cause NFs to accumulate in the perikaryon (25, 26) is supported by axonal transport studies. There are several reports of an apparent correlation between extensive tail domain phosphorylation and a reduction in the rate of NF transport (67–69). The premature phosphorylation of KSP repeats in the perikaryon might interfere with the association between NFs and components involved in their axonal transport. Since the latter may include microtubules, the observation that NFH tail domain phosphorylation favors the dissociation of NFH from microtubules (70) could explain how aberrant NF phosphorylation might promote perikaryal accumulation.

Several recent transgenic mouse studies indicate that perturbations in NF homeostasis brought about by mutation, or overexpression of individual NF subunits, can cause pathological neurofilamentous accumulations in neuronal cell bodies (71–74). Whether there is a similar causal relationship between hyperphosphorylation and accumulation of perikaryal NFs remains to be determined. The finding that some sporadic amyotrophic lateral sclerosis patients have NFH alleles with deletions in the KSP repeat domain suggests that altered phosphorylation may indeed be a cause of neurofilamentous accumulations (75, 76). Our demonstration that mechanical disruption of neurites in DRG cultures caused the hyperphosphorylation of perikaryal NFs reproduced the effects of axonal injury seen in animal studies (50). Again this finding implicates stress-activated pathways, this time in response to mechanical injury of axons.

The demonstration that a SAPK(s) is involved in the phosphorylation of perikaryal NFs provides the basis for studies to determine whether aberrant phosphorylation has deleterious effects on neuronal integrity. Our findings suggest that the

presence of hyperphosphorylated NFs in the neuronal perikaryon can serve as a marker for SAPK activation. This type of basic information may lead to a better understanding of the etiology of several neurological diseases.

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Chapter 6

Study of Proline-directed Protein Kinases Involved in Phosphorylation of the Heavy Neurofilament Subunit (NFH)

Abstract

The high molecular mass neurofilament subunit (NFH) is normally hypophosphorylated in the neuronal perikaryon and undergoes extensive phosphorylation upon entering the initial axon segment. Aberrant hyperphosphorylation of perikaryal NFH is a common feature of many neurological diseases. In a previous study (Giasson and Mushynski, 1996), we demonstrated a correlation between phosphorylation of perikaryal NFH and induction of stress-activated protein kinase (SAPK)-γ. In this report, we present direct evidence showing that the *in vivo* activation of SAPKs by an upstream activator (MEKK-1) causes extensive NFH phosphorylation. We also show that stress activated-p38 kinases are not involved in the phosphorylation of perikaryal NFH in cultured dorsal root ganglion neurons and that this process is reversible. SAPKγ is shown to be located both in the cell body and neurites of the cultured neurons, suggesting that it is likely to be involved in the phosphorylation of cytoplasmic substrates. These could include neuritic NFH, which is highly phosphorylated despite the demonstrated lack of cyclin-dependent kinase-5 activity in these neurons. Neuritic NFH is also highly phosphorylated in neuronal cultures devoid of Schwann cells, indicating that this form of post-translational modification does not require cues stemming from Schwann cell-axon contacts. Collectively, these findings provide significant new insights into mechanisms involved in NFH phosphorylation in normal neurons and in disease states characterized by aberrant phosphorylation of neurofilaments.

Introduction

Neurofilaments (NFs) are the principal intermediate filaments (IFs) found in many types of mature neurons. They are the most abundant structure in large myelinated axons (Hoffman et al., 1984), and are an important determinant of axonal caliber (Yamasaki et al., 1991; Ohara et al., 1993; Eyer and Peterson, 1994). NFs are composed of three proteins, the low- (NFL), mid-sized- (NFM), and heavy- (NFH) molecular mass subunits (Hoffman and Lasek, 1975). In common with other IF proteins, each NF subunit contains a highly conserved α-helical rod domain, involved in dimer formation, flanked by an amino-terminal head domain and a carboxy-terminal tail domain (Fuchs and Weber, 1994).

NFH from myelinated axons is highly phosphorylated *in vivo* (Julien and Mushynski, 1982), predominantly at Lys-Ser-Pro (KSP) repeats in the tail domain (Julien and Mushynski, 1983; Lee et al., 1988; Elhanany et al, 1994). The role of NFH tail domain phosphorylation is not fully understood although it has been shown to inhibit interaction between NFH and microtubules (Hisanaga et al., 1991, 1993a, b; Miyasaka et al, 1993) and to protect NFH from proteolysis (Goldstein et al, 1987; Pant, 1988). It may also regulate the distribution of NFs between stationary and mobile phases in the axon (Lewis and Nixon, 1988).

The use of monoclonal antibodies that could distinguish between phosphorylated and unphosphorylated epitopes in the tail domain of NFH has shown that axonal NFH is normally more highly phosphorylated than that located in the cell body and dendrites (Sternberger and Sternberger, 1983; Lee et al., 1987). Perikaryal NFH is maintained in a hypophosphorylated state with an apparent molecular mass of 160 kDa on SDS-polyacrylamide gel electrophoresis (PAGE), compared to a value of 200 kDa for axonal NFH (Glicksman et al., 1987; Oblinger, 1987; Nixon et al., 1989). The gel electrophoretic mobility of axonal NFH increases to that of perikaryal NFH following dephosphorylation of the tail domain (Julien and Mushynski, 1982; Carden et al., 1985) and this shift is reversed by phosphorylation at KSP repeats (Hisanaga et al 1991, 1993b ; Miyasaka et al., 1993). Of the neuronal proline-directed protein kinases that can phosphorylate NFH only tau protein kinase II/cyclin-dependent kinase-5 (cdk-5) has been shown unequivocally to cause a reduction in its mobility on SDS-PAGE to levels seen for axonal NFH (Hisanaga et al., 1993b; Kobayashi et al., 1993; Miyasaka et al., 1993; Guidato 1996a; Sun et al., 1996).

Perikaryal NFH is highly phosphorylated in many neurodegenerative diseases, such as Alzheimer's (Cork et al., 1986; Zhang et al., 1989), Parkinson's (Forno et al.,

1986; Pollanen et al., 1994), and amyotrophic lateral sclerosis (ALS) (Manetto et al., 1988; Munoz et al., 1988; Sobue et al., 1990). We previously presented correlative evidence indicating that stress-activated protein kinase γ (SAPKγ) could be responsible for the aberrant phosphorylation of perikaryal NFH (Giasson and Mushynski, 1996). SAPKs are proline-directed kinases belonging to the mitogen-activated protein (MAP) kinase family, which also includes extracellular signal-regulated kinases (ERKs), p38 kinases (Cano and Mahadevan, 1995; Kyriakis and Avruch, 1996) and a novel member, SAPK-3 (Mertens et al., 1996). The MAP kinases are related structurally and are activated by similar cascades in response to diverse stimuli (Cano and Mahadevan, 1995; Kyriakis and Avruch, 1996).

In this report, we present direct evidence that the *in vivo* activation of SAPKs by constitutively active MAP kinase/ ERK kinase kinase-1 (MEKK-1) induces phosphorylation of the NFH tail domain. We also show that p38 kinases are not involved in the hyperphosphorylation of perikaryal NFH and that this process is completely reversible. These findings provide basic information that enhances our understanding of mechanisms causing aberrant NF phosphorylation in neurological diseases.

Materials and Methods

Materials

Nerve growth factor (NGF) (2.5S) was purchased from Prince Laboratories (Toronto, ON). Anti-NF antibodies SMI 31 and SMI 34 were obtained from Sternberger Monoclonals, Inc. (Baltimore, MD). Anti-SAPKγ (C-17), anti-ERK-1 (C-16), anti-ERK-2 (C-14), anti-p38α (C-20), anti-cdk-5 (C-8) polyclonal antibodies, anti-cdk-5 (DC17) monoclonal antibody and glutathione S-transferase (GST)-c Jun (amino acids 1-79) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Histone H1 was obtained from Life Technologies, Inc. The pRC/CMV eukaryotic expression vector was purchased from Invitrogen (San Diego, CA). Anti-NFH (N52) and anti-NFL (NR4) monoclonal antibodies were from Sigma. Polyclonal anti-vimentin antibody and N-acetyl-Leu-Leu-norleucinal (CI) were from ICN (Mississauga, ON). SB 203580 was generously provided by SmithKline Beecham.

Cell Culture

Embryonic day 15-16 dorsal root ganglia (DRGs) were dissected, dissociated and maintained in culture as previously described (Giasson and Mushynski, 1996). In order to allow for the manual separation of cell bodies from neurites, the dissociated DRGs were plated in a small area at the center of a 35 mm culture dish. Neurites extended radially to form a halo surrounding the cell body mass. For cultures treated with anti-mitotic agents, the cells were cycled between 10^{-5} M 5-fluoro-2'-deoxyuridine/ 10^{-6} M cytosine β-D-arabino-furanoside and $5x10^{-6}$ M 5-fluoro-2'-deoxyuridine/ $5x10^{-7}$ M cytosine β-D-arabino-furanoside every 4 days for 16 days, starting 24 h after plating.

NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in 85% Dulbecco's modified Eagle's medium (high glucose), 10% heat-inactivated horse serum, 5% fetal bovine serum (Life Technologies, Inc.) and antibiotics. The cells were transfected using lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer's instructions.

Immunoprecipitation Kinase Assays

SAPKγ activity was assayed as previously described (Giasson and Mushynski, 1996). Briefly, following cell lysis in the presence of Triton X-100, cell debris was removed by centrifugation at 13,000xg, and the protein concentration of each supernatant was determined to equalize the amount of protein used in each immunoprecipitation. SAPKγ was immunoprecipitated, the immunoprecipitates were washed extensively, and activity was assayed using [γ^{32}P]-ATP and GST-cJun as a substrate. Phosphorylation of GST-cJun was visualized after SDS-PAGE (Laemmli, 1970) by autoradiography of dried gels and quantified using a Fujix BAS2000 Bio-Imaging Analyzer (Fuji Bio-Imaging).

Cdk-5 activity was assayed by immunoprecipitation kinase assay, as previously described (Tsai et al., 1993), using an anti-cdk-5 polyclonal antibody (C-8) and histone H1 as the substrate. Visualization of the phosphorylated substrate was achieved as described for SAPKγ.

Gel Electrophoresis and Western Blot Analysis

Cells were harvested in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), lysed in 2% SDS, 62.5 mM Tris, pH 6.8, and protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce). Glycerol and β-mercaptoethanol were added to concentrations of 10% and 5%, respectively. The cell extracts were diluted to the appropriate concentrations with SDS-sample buffer (2% SDS, 62.5 mM Tris, pH 6.8, 10% glycerol and 5% β-mercaptoethanol) and the proteins were resolved on slab gels by SDS-PAGE (Laemmli, 1970). Proteins were electrophoretically transferred to Immobilon-P membrane (Millipore Corp.) in buffer containing 48 mM Tris, 39 mM glycine and 5% methanol. The membranes were blocked with 1% skimmed milk powder in Tris-buffered saline/Tween (20 mM Tris, pH 7.7, 137 mM NaCl, and 0.1 % Tween 20), incubated with primary antibodies and developed using the ECL Western Blotting Detection Kit (Amersham Corp.).

Results

Transfection of cells with MEKK-1Δ induces NFH tail domain phosphorylation.

MEKK-1Δ, a constitutively active form of MEKK-1 which serves as an activator of the SAPK cascade (Minden et al., 1994; Yan et al., 1994; Xu et al., 1995), was tested for its ability to induce NFH phosphorylation *in vivo*. NIH 3T3 cells transfected with the expression vector pRC/CMV alone did not express NFH (Fig. 1A, lanes 1). In extracts from cells transfected with the expression vector containing the mouse NFH gene (Julien et al., 1988) beginning 15 nucleotides upstream from the translational start site, NFH was detected with N52 antibody as a predominantly hypophosphorylated isoform(s), judging from its mobility on SDS-PAGE and from its failure to bind monoclonal antibodies SMI 31 or SMI 34 (Fig.1A, lanes 2). Monoclonal antibody N52 can detect both hypo- and hyperphosphorylated forms of NFH (Shaw et al., 1986), although the relevant epitope can be blocked by cdk-5 phosphorylation (Guidato et al., 1996b). SMI 31 and SMI 34 are both phosphorylation-dependent monoclonal antibodies that react with different epitopes in the tail domain of NFH (Sternberger and Sternberger, 1983; Lee et al., 1988; Shea and Beermann, 1993). Co-transfection of NIH 3T3 cells with pRC/CMV vectors expressing NFH and MEKK-1Δ yielded hyperphosphorylated NFH, as determined by its reduced

mobility on SDS-PAGE and by its reactivity with both SMI 31 and SMI 34 (Fig.1A, lanes 3). The expression of MEKK-1Δ also resulted in the activation of SAPKγ (Fig. 1B).

P38 kinases are not involved in stress-induced NFH phosphorylation.

Proline-directed p38 kinases are often activated simultaneously with SAPKs (Cano and Mahadevan, 1995; Raingeaud et al., 1995). To test whether p38 kinases are also involved in the hyperphosphorylation of perikaryal NFH, we used a specific inhibitor, SB 203580 (IC_{50}=0.6 μM), which does not inhibit SAPKs (Cuenda et al., 1995). Cultured DRG neurons were treated with 30 μM CI, a calpain (Saito and Nixon, 1993) and proteasome inhibitor (Tsubuki et al., 1993; Rock et al., 1994), which has been shown to activate SAPK and induce hyperphosphorylation of perikaryal NFH (Giasson and Mushynski, 1996) (Fig. 2, lane 2). The addition of 20 μM SB 203580 had no effect on the CI-induced reduction in mobility, and hence phosphorylation, of NFH (Fig. 2, lane 3).

Distribution of MAP kinases in DRG neurons

The distribution of MAP kinases within DRG neurons was assessed by Western blot analysis as shown in Fig. 3. DRG cultures maintained in medium containing anti-mitotic agents were fractionated into neurite- (lanes 1) and cell body- (lanes 2) enriched fractions as described in "Materials and Methods". The anti-mitotic agents eliminated all of the Schwann cells normally found in DRG cultures and prevented the proliferation of fibroblasts. However, the cultures still contained a population of quiescent fibroblasts resistant to anti-mitotic treatment. To compensate for contamination by these fibroblasts, we prepared DRG cultures treated with anti-mitotic agents and maintained without NGF to eliminate neurons (Giasson and Mushynski, 1997). Lane 3 in Fig. 3 was loaded with an amount of protein from fibroblast cultures equal to that for neurite- (lanes 1) and cell body- (lanes 2) enriched fractions. Lanes 4, 5 and 6 were respectively loaded with two-, four-, and eight- fold less fibroblast protein than lane 3. The inclusion of lanes 3-6 allowed us to determine whether the proteins detected in lanes 1 and 2 were neuronal in origin or from contaminating fibroblasts. Vimentin and NFL were used as specific markers for fibroblasts and DRG neurons, respectively. There were equivalent amounts of NFL in the neuronal cell body- and neurite- fractions, and NFL was not detected in cultures maintained without NGF. Two other DRG neuronal markers, peripherin and α-internexin (Athlan et al., 1997), were also not detected in the fibroblast cultures (data not shown). There were

approximately equal levels of fibroblast contamination in the neuronal cell body and neurite fractions as determined by their vimentin content, and these fractions contained less than 12% fibroblast protein. P38α was expressed at low levels in DRG neurons and only in the cell body fraction. ERK-1/-2 and SAPKγ were equally distributed between the cell body and neurite fractions.

The hyperphosphorylation of perikaryal NFH is reversible

Cultured DRG neurons were treated with 30 μM CI to induce the hyperphosphorylation of perikaryal NFH (Giasson and Mushynski, 1996), as reflected in its reduced mobility on SDS-PAGE (Fig. 4, lane 2). Following removal of CI from the culture medium, perikaryal NFH was seen to undergo progressive dephosphorylation. Approximately half of the protein had returned to its normal mobilty on SDS-PAGE within 2 days (Fig. 4, lane 3); by 4 days almost all of the NFH had return to a normal hypophosphorylated state (Fig. 4, lane 4).

Axonal NFH in DRG neurons is hyperphosphorylated despite the inactivity of cdk-5

The Western blots in Figure 5A show that most of the NFH in the neuronal cell body-enriched fraction was hypophosphorylated, while that in the neurite-enriched fraction was mostly hyperphosphorylated. The small amount of hypophosphorylated NFH in the neurite-enriched fraction originates from neuronal cell bodies localized outside of the circumference of the circular punch used to separate the two neuronal compartments. The hyperphosphorylated NFH in cell body- enriched extracts derives from the initial segment of neurites and from neurites criss-crossing the area occupied by the cell body mass. The slowly migrating, highly phosphorylated isoforms of NFH reacted with both phosphorylation dependent antibodies, SMI 31 and SMI 34. Hence, NFH in cultured DRG neurons demonstrated the normal phosphorylation pattern (Sternberger and Sternberger, 1983; Glicksman et al., 1987; Lee et al., 1987; Oblinger, 1987; Nixon et al., 1989), which was also observed in DRG cultures treated with anti-mitotic agents and devoid of Schwann cells (Fig. 5B).

The activity of cdk-5 in cultured DRG neurons was determined by immunoprecipitation kinase assays using rat brain extract as a positive control (Fig. 6A, lanes 1 to 6)(Tsai et al., 1993). Since the relative amounts of immunoprecipitable cdk-5 in brain extract as compared to extract from DRG cultures were unknown, different amounts of rat brain extract were used in the immunoprecipitation kinase assays, and levels of immunoprecipitated cdk-5 were determined by Western blot analysis (Fig. 6B). Despite the

fact that comparable amounts of cdk-5 were immunoprecipitated from 200 μg of DRG extract and 100 μg of brain extract (Fig. 6B, lanes 4 and 8), no histone H1 phosphorylating activity was detected in DRG samples (Fig. 6A, lane 8). Western blot analysis of total protein extracts from rat brain and DRG cultures revealed that on an equal protein basis, rat brain contained approximately twice as much cdk-5 as did the DRG cultures (data not shown).

Discussion

This study presents direct evidence that SAPKs can phosphorylate the tail domain of NFH, as reflected both in the reduced mobility of NFH on SDS-PAGE and in its immunoreactivity with the phosphorylation-dependent monoclonal antibodies, SMI 31 and SMI 34 (Fig.1). SAPK activation was accomplished by transfection of a vector expressing constitutively active MEKK-1Δ, which activates JNK kinase (JNKKMKK4/SEK4), the upstream regulator of SAPKs (Yan et al., 1994; Dérijard et al., 1995; Lin et al., 1995). Although MEKK1 can also activate the ERK pathway (Lange-Carter and Johnson, 1994; Xu et al., 1995) it is a more efficient activator of the SAPK cascade (Minden et al., 1994; Yan et al., 1994). Furthermore, we previously demonstrated that ERK activation did not result in a detectable increase in the *in vivo* phosphorylation of NFH (Giasson and Mushynski, 1996) and others have shown that the *in vitro* phosphorylation of NFH by ERKs did not cause a significant reduction in its mobility on SDS-PAGE (Roder and Ingram, 1991; Roder et al., 1995). Use of a specific inhibitor of p38 kinases, SB 203580, demonstrated that the latter enzymes are not involved in the hyperphosphorylation of perikaryal NFH (Fig. 2). These results support our previously reported correlative study (Giasson and Mushynski, 1996) and strongly suggest that SAPKs are involved in the aberrant phosphorylation of perikaryal NFH.

The stress activated phosphorylation of perikaryal NFH is completely reversible (Fig. 4), indicating that a protein phosphatase(s) in the neuronal perikaryon can maintain the protein in a hypophosphorylated state. A protein phosphatase-2A-like activity has been reported to dephosphorylate KSP repeats in NFH (Veeranna et al., 1995). However, attempts to dephosphorylate NFH *in vitro* to an extent that would alter its electrophoretic mobility using either of the major neuronal protein phosphatases, 1, 2A, 2B and 2C, were unsuccessful (Hisanaga et al., 1993a). This discrepancy may be due to differences between the *in vivo* and *in vitro* conformations of NFH, to differences in substrate specificity conferred by regulatory subunits associated with the catalytic phosphatase subunit (Sola et

al., 1991), or to the involvement of a different protein phosphatase such as PP-X (Brewis et al., 1993). In any case, our results are consistent with the presence of an NFH tail domain phosphatase in the neuronal perikaryon. This enzyme may be absent from or less active in axons, where NFH is highly phosphorylated.

We have also demonstrated that ERKs and SAPKγ are equally distributed between the cell body and neurite compartments of DRG neurons. The localization of ERKs in neurites is consistent with a recent study demonstrating the axonal transport of these enzymes (Johanson et al., 1995). Although SAPKs have been reported to phosphorylate transcription factors primarily (Cano and Mahadevan, 1995; Kyriakis and Avruch, 1996), the axonal localization of SAPKγ suggests that it may also be involved in the phosphorylation of cytoplasmic substrates, such as NFH.

Cdk-5 is the only neuronal kinase (Hisanaga, 1993b; Miyasaka et al., 1993), other than SAPKs, that has been shown to phosphorylate NFH to the point of reducing its mobility on SDS-PAGE to that of axonal NFH. Cdk-5 has been reported to phosphorylate NFH *in vitro* to the extent of 3-5 (Miyasaka et al., 1993) or 10 (Hisanaga et al., 1993b) moles of phosphate per mole of NFH, preferentially at KSPXK repeats (where X is not an acidic residue) (Beaudette et al., 1993). We have observed that neuritic NFH in cultured DRG neurons is highly phosphorylated despite the demonstrated lack of cdk-5 activity (Fig. 6), which is likely to be due to the fact that its activator ligand, p35/p25 (Tsai et al., 1994; Lew et al., 1994) is not expressed in these neurons (Tsai et al., 1994). This may explain the apparent sparing of DRG neurons in cdk-5 deficient mice whereas many types of CNS neurons in these animals are adversely affected (Ohshima et al., 1996). Consequently, it is possible that KSPXK motifs are not phosphorylated in DRG neurons, as is suggested by the finding that NFH is more highly phosphorylated in ventral root motor neurons than in dorsal root neurons (Soussan et al., 1996). Cdk-5 is likely to be active in and required for motor neuron survival since the latter show a number of abnormalities in mice lacking the enzyme, including ballooned perikarya, dispersed Nissl substance and cytoplasmic vacuoles (Ohshima et al., 1996).

NFH is reported to undergo high levels of phosphorylation in the initial axon segment, at the site where myelination begins (Hsieh et al., 1994, Nixon et al., 1994a). We have demonstrated that NFH follows the normal pattern of phosphorylated isoform distribution in cultured DRG neurons despite the lack of myelination (Fig. 5A). Furthermore, cultures devoid of Schwann cells also exhibited the normal NFH phosphorylation profile (Fig. 5B). These experiments clearly demonstrate that phosphorylation of neuritic NFH is not initiated solely by cues stemming from Schwann

cell-axon interactions. As mention above, there may be differences in phosphatase activity levels between the cell body and neuritic compartments. However, it is also likely that NFH-kinase(s) is (are) activated in the initial axon segment and these enzymes could conceivably include SAPKs.

Phosphorylation of the NFH tail domain does not occur exclusively during the entry of NFs into axons. Phosphate addition continues during NF transport (Lewis and Nixon, 1988; Archer et al., 1994; Nixon et al, 1994b) and regional differences in tail domain phosphorylation within myelinated axons have also been reported. There is a reduced level of NF phosphorylation at the node of Ranvier (Mata et al., 1992) and a decrease in axonal NFH phosphorylation in hypomyelinating transgenic or Trembler mice (deWaegh et al., 1992; Cole et al., 1994). The latter observation indicates that axonal properties, including NFH phosphorylation, are modulated by signals transmitted from myelinating Schwann cells to axons. NFH phosphorylation in myelinated regions may thus be augmented through the activation of proline-directed kinases such as ERKs and SAPKs.

There is evidence suggesting that aberrant NF metabolism may be involved in the etiology of ALS (Côté et al., 1993; Xu et al., 1993). Motor neurons containing abnormally hyperphosphorylated perikaryal NFH and proximal axonal enlargements filled with NFs are characteristic of the disease (Carpenter, 1968; Hirano et al., 1984; Manetto et al., 1988; Munoz et al., 1988; Sobue et al., 1990). Furthermore, ALS is a neurodegenerative disease that targets the large, NF-rich motor neurons predominantly and large sensory neurons to a lesser degree (Tsukagoshi et al., 1979; Kawamura et al., 1981). If NFs are involved in ALS pathogenesis, it is more likely due to an impairment of axonal transport rather than simple accumulation of NFs in the perikaryon (Collard et al., 1995; Marszalek et al., 1996).

The mechanism underlying the axonal transport of NFs remains unsettled, although experiments with the neurotoxin, β,β'-iminodipropionitrile (IDPN), suggest that microtubules may be involved. IDPN causes NFs and microtubules to segregate (Griffin et al., 1978; Papasozomenos et al., 1981) and at appropriate doses, blocks NF movement but has only a modest effect on the transport of microtubules (Griffin et al., 1978). This causes large masses of NFs to accumulate in the proximal axon (Chou and Hartman, 1965). It is interesting to note in this regard that NFH interacts with microtubules only when its tail domain is hypophosphorylated (Hisanaga et al., 1991; 1993a, b; Miyasaka, 1993), suggesting that tail domain phosphorylation may be important in regulating the transport of NFs from the cell body to the axon. Aberrant hyperphosphorylation of perikaryal NFH in

neurons subjected to some form of stress may thus be responsible for the formation of neurofilamentous accumulations that characterize many neurological diseases.

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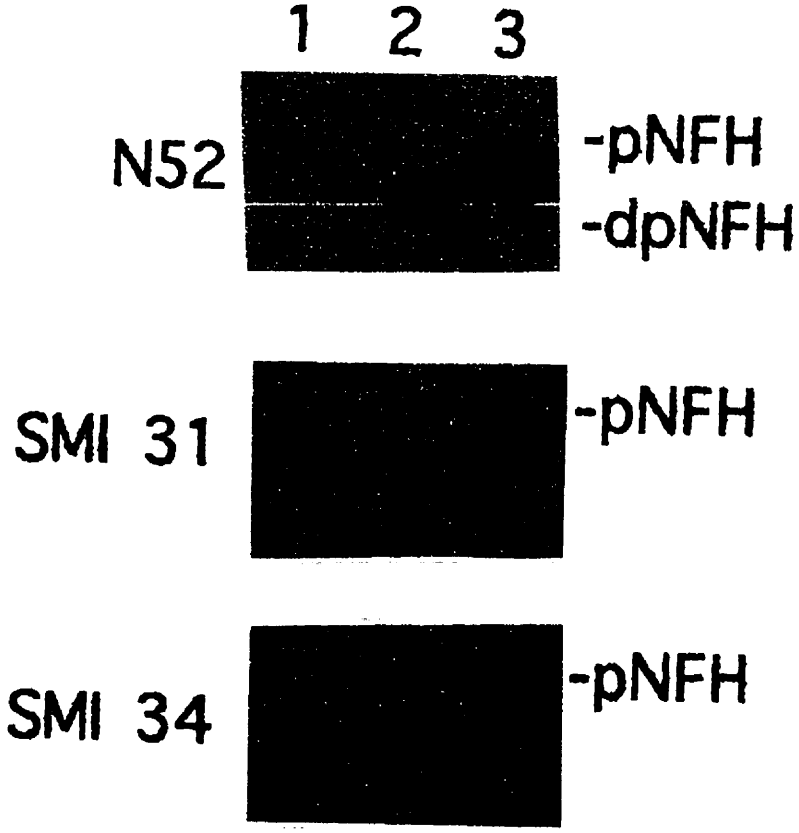
Figures

Figure 1. Transient transfection with constitutively active MEKK-1Δ induces NFH tail domain phosphorylation. NIH 3T3 cells were transfected with the pRC/CMV eukaryotic expression vector (lanes 1), with the mouse NFH gene cloned into the pRC/CMV vector (lanes 2) or both the mouse NFH gene and MEKK-1Δ cDNA, each cloned into pRC/CMV (lanes 3). (A) NFH was detected by Western blot analysis using monoclonal antibodies N52, SMI 31 or SMI 34. pNFH and dpNFH refer to hyper- and hypophosphorylated NFH, respectively. Equal amounts of protein were loaded in each lane. (B) The activity of SAPKγ was determined by immunoprecipitation kinase assays as described in "Materials and Methods". ^{32}P-phosphorylation of GST-cJun was visualized by autoradiography and quantified by image analysis. The relative activity of the immunoprecipitated kinase is indicate below each lane.

A


1 2 3
N52
-pNFH
-dpNFH
SMI 31
-pNFH
SMI 34
-pNFH

B

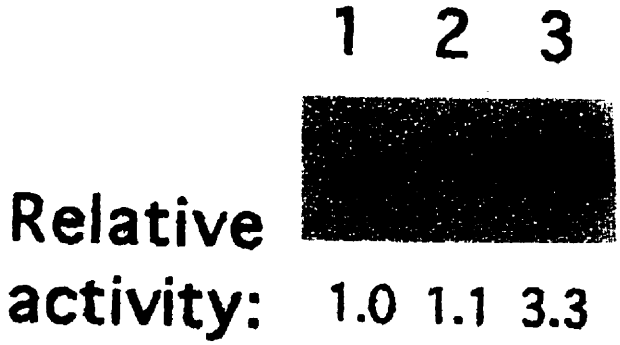

1 2 3
Relative activity:
1.0 1.1 3.3

Figure 2. P38 kinases are not involved in perikaryal NFH hyperphosphorylation. Localized DRG cultures were prepared as described under "Materials and Methods". The cultures were maintained for 20 days (lane 1, control) and treated with 30 μM CI for 10 h (lane 2). A culture was pretreated with 20 μM SB 203580 for 2 h, prior to the addition of 30 μM CI for 10 h (lane 3). The neuronal cell bodies were manually separated from the neurites and subjected to Western blot analysis using anti-NFH monoclonal antibody, N52. pNFH and dpNFH refer to hyper- and hypophosphorylated NFH, respectively.

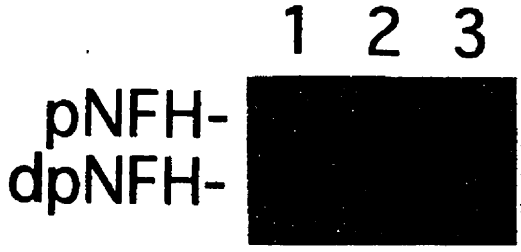

1 2 3
pNFH-
dpNFH-

Figure 3. SAPKγ and ERKs are located in both perikaryon and neurites of DRG neurons. DRG neurons were maintained in culture in the presence of anti-mitotic agents as described in "Material and Methods". The cultures were separated into neurite- (lanes 1) and cell body- (lanes 2) enriched fractions. Protein extracts from DRG cultures maintained with anti-mitotic agents and without NGF were loaded in lanes 3 to 6. Lanes 1, 2 and 3 were loaded with 5 μg of protein, while lanes 4, 5 and 6 were loaded with 2.5 μg, 1.25 μg, and 0.62 μg of protein, respectively. The proteins were detected by Western blot analysis. NFL, Vim, SAPKγ, ERK 1,2 and p38 refer to the low molecular mass neurofilament subunit, vimentin, SAPKγ, ERK-1/-2. and p38α kinase, respectively.

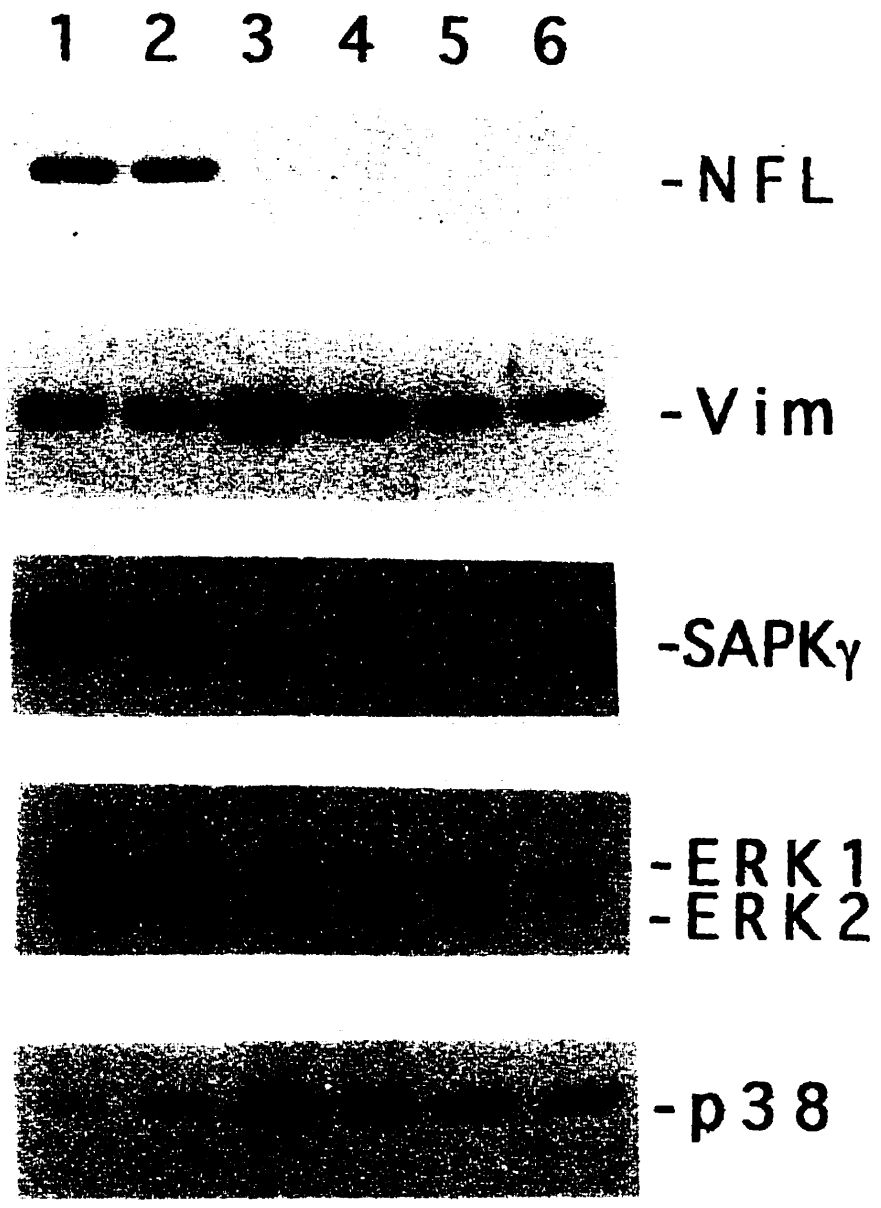

1 2 3 4 5 6
-NFL
-Vim
-SAPKγ
-ERK1
-ERK2
-p38

Figure 4. Aberrant phosphorylation of perikaryal NFH is reversible. Localized DRG cultures were prepared as described under "Materials and Methods". The cultures were maintained for 20 days (lane 1) and treated with 30 μM CI for 10 h (lanes 2-6). Following treatment with CI the cultures were maintained in CI-free medium for 2 days (lane 3), 4 days (lane 4), 6 days (lane 5), and 8 days (lane 6). The neuronal cell bodies were manually separated from the neurites and subjected to Western blot analysis using anti- NFH monoclonal antibody, N52. pNFH and dpNFH refer to hyper- and hypophosphorylated NFH, respectively.

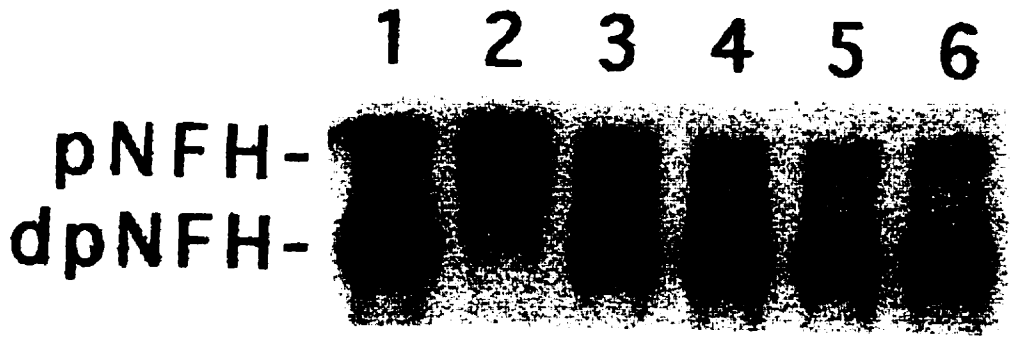

1 2 3 4 5 6
pNFH-
dpNFH-

Figure 5. Distribution of phosphorylated NFH isoforms in DRG neurons. Localized DRG cultures were prepared as described under "Materials and Methods" and separated into cell body- (C) and neurite-enriched (N) fractions. The two subcellular fractions were subjected to Western blot analysis using anti-NFH monoclonal antibodies N52, SMI 31 and SMI 34. pNFH and dpNFH refer to hyper- and hypophosphorylated NFH, respectively. (A) DRG cultures were maintained for 20 days. Cell body- and neurite-enriched fractions were harvested in equal volumes of SDS-sample buffer and the same volume was loaded in each lane. (B) DRG cultures were maintained in the presence of anti-mitotic agents as described in "Material and Methods". Cell body- and neurite-enriched fractions were lysed in 2% SDS, 62.5 mM Tris, pH 6.8, protein concentrations were determined and equal amounts of protein were loaded in each lane.

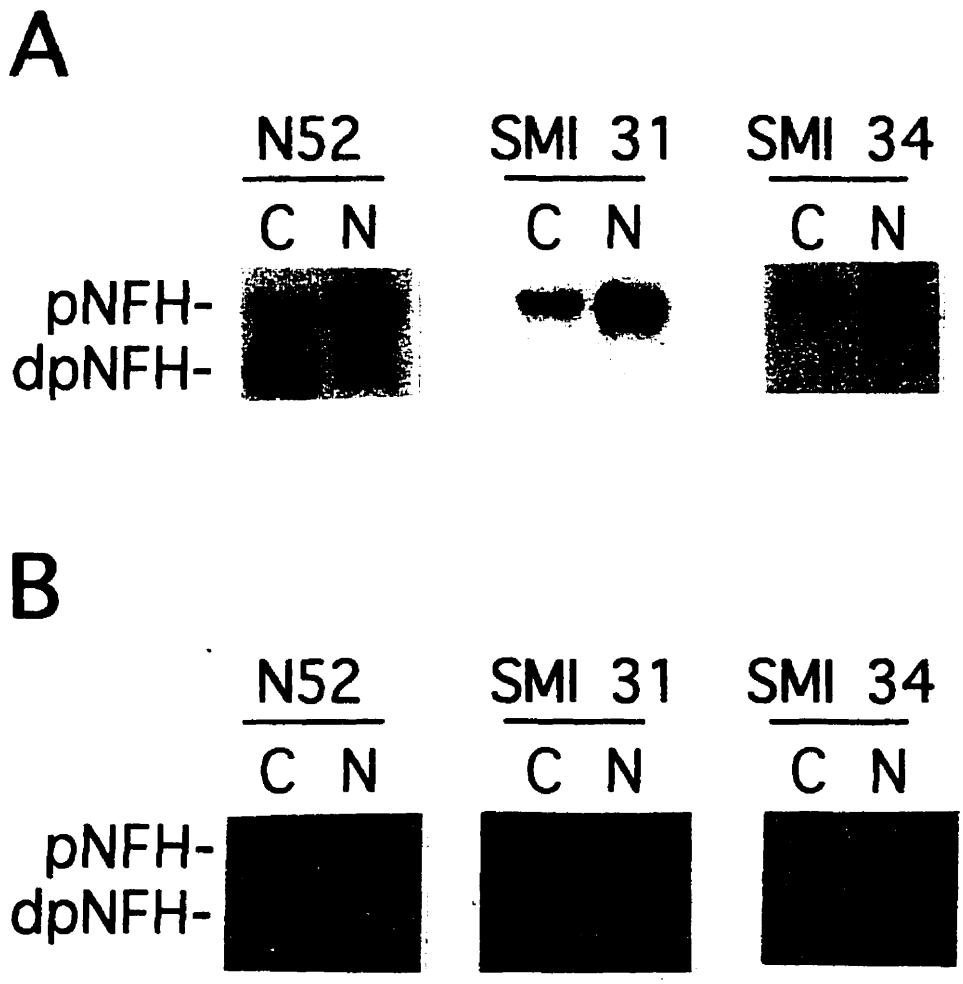
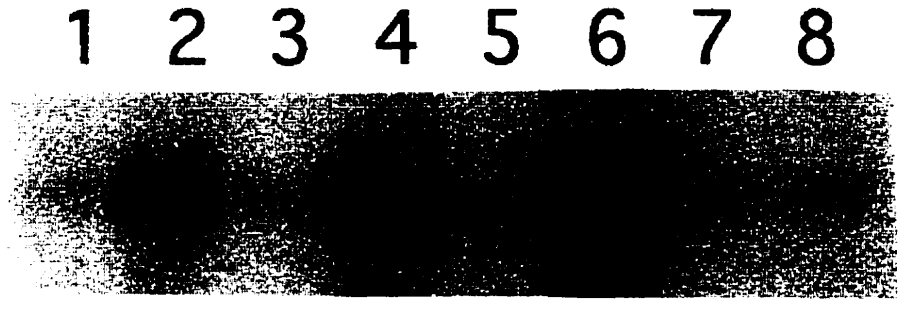
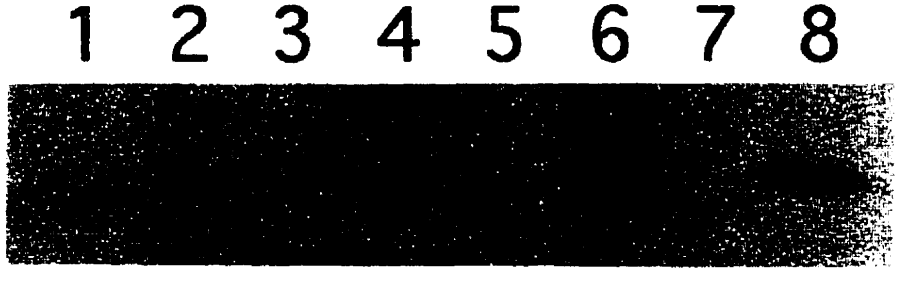

A
N52
SMI 31
SMI 34
C N
C N
C N
pNFH-
dpNFH-
B
N52
SMI 31
SMI 34
C N
C N
C N
pNFH-
dpNFH-

Figure 6. Analysis of anti-cdk-5-immunoprecipitable histone H1 kinase activity from DRG cultures and adult rat brain. (A) Fifty (lanes 1 and 2), 100 (lanes 3 and 4) and 200 (lanes 5 and 6) μg of protein from brain and 200 μg of protein from DRG cultures maintained for 20 days (lanes 7 and 8) were immunoprecipitated with non-immune serum (lanes 1, 3, 5 and 7) and anti-cdk-5 (C-8) polyclonal antibody (lanes 2, 4, 6 and 8). The activity of cdk-5 was assayed with [γ-^{32}P]-ATP and histone H1. (B) The cdk-5 immunoprecipated from (A) was detected by Western blot analysis using anti-cdk-5 monoclonal antibody (DC17).

A



B



Chapter 7

General Discussion

The work presented in this thesis has significantly advanced our knowledge of neuronal IF phosphorylation and dynamics. When these studies were initiated, there was compelling evidence to support the notion that phosphorylation by $p34^{cdc2}$ kinase was involved in the rearrangement and disassembly of types III and V IFs during cell division (see section 1.2.4). On the other hand, *in vivo* evidence that neuronal IF assembly could be regulated by phosphorylation was limited to studies showing that inhibition of protein phosphatases, by either okadaic acid or calyculin A, caused NF fragmentation in DRG neurons (Sacher et al., 1992; 1994). The only other reported results linking phosphorylation to the regulation of neuronal IF assembly were performed *in vitro* with purified α-internexin and PKA (Tanaka et al., 1993), or with NFL and PKA or protein kinase C (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990). The *in vitro* phosphorylation of α-internexin or NFL by these respective kinases blocked their polymerization and caused homopolymeric filaments to disassemble.

The relevance of phosphorylation experiments performed with purified NFL became somewhat questionable, when it was shown that NFs are obligate heteropolymers *in vivo* (Ching and Liem, 1993; Lee et al., 1993). The *in vivo* assembly of NFs requires NFL and a substoichiometric amount of either NFM or NFH. The vulnerability of homopolymeric NFL filaments to phosphorylation by either PKA or protein kinase C may thus be due to an inherent instability of these filaments which are assembled *in vitro* under optimized conditions. This concern was further substantiated when it was shown that native NFs or reassembled NFs are much more resistant to disassembly due to PKA phosphorylation than NFL homopolymers (Hisanaga et al., 1994).

The results presented in Chapters 2 and 3 of this thesis clearly demonstrate that the assembly state of neuronal IFs can be modulated by phosphorylation *in vivo*. These results also indicate that, at least in DRG neurons, PKA and PP-2A can regulate the assembly of neuronal IFs. The phosphorylated amino acid residues involved in the latter regulation are located in the head domain of NFL and/or peripherin, and perhaps of NFM as well. Due to the large number of phosphorylation sites in peripherin and NFM, it is difficult to determine which ones are responsible for the disassembly process. Site-directed mutagenesis will no doubt help in this determination once a suitable neuronal system for transfection studies become available.

The simultaneous inhibition of PP-2A was required for the activation of PKA to produce a detectable change in the assembly state of neuronal IFs in cultured DRG neurons

(see Chapters 2 and 3). This result suggests that the action of PP-2A predominates over that of PKA. However, it is also possible that PKA activation alone may cause changes in the assembly state of neuronal IFs that cannot be detected by the methods used. Furthermore, even though extensive cytoskeletal rearrangements such as those reported in Chapter 2 and 3 are unlikely to occur under normal circumstances, more moderate and localized changes of a similar nature are likely taking place during the normal course of neuronal IF metabolism.

The results in Chapter 3 demonstrate that phosphorylation of α-internexin is insignificant when neuronal IF fragmentation is induced by the simultaneous activation of PKA and inhibition of PP-2A in DRG neurons.The low level of phosphate incorporation into α-internexin contrasts with the levels observed in peripherin and NF proteins. The lack of any correlation between α-internexin phosphorylation and PKA-induced IF disassembly sheds doubt on the physiological relevance of the *in vitro* results obtained with purified α-internexin and PKA (Tanaka et al., 1993). At the very least, it emphasizes the need for caution in interpreting the results of *in vitro* experiments performed with purified IF proteins and kinases.

Chapter 4 provides the first experimental evidence supporting the notion (Nixon and Shea, 1992) that α-internexin is expressed earlier than other neuronal IF proteins to provide a more flexible IF network during development. As mentioned earlier, phosphorylation of α-internexin is not involved in neuronal IF dynamics in DRG neurons, although its presence as an integral constituent of this IF network (Athlan et al., 1997) may render it more flexible and perhaps more susceptible to phosphorylation-induced disassembly. The latter possibility may explain the difference in sensitivity of purified, native NFs (Hisanaga et al., 1994) and the neuronal IFs in DRG neurons to phosphorylation-induced disassembly. Experiments performed with native NFs prepared from adult animals are likely to contain a much lower ratio of α-internexin to NF proteins than the IF network in cultured neurons from E15-E16 DRGs since α-internexin is down-regulated and NFs are upregulated with age (see section 1.3). However, the possibility that peripherin, which is present in DRG neurons but not in spinal NF preparations, may also contribute to the different properties of the two IF networks cannot be ruled out. Furthermore, it is possible that the structure and/or properties of NFs may be altered by the procedures used in their purification.

The results presented in Chapters 5 and 6 demonstrate that activation of SAPKs causes hyperphosphorylation of perikaryal NFH. These findings strongly support the notion that aberrant phosphorylation of perikaryal NFH reported in many disease states is due to the activation of SAPKs and not to a NF transport problem. It had been speculated that, because NFH is normally hyperphosphorylated at the initial axonal segment, impairment of NF transport may lead to the accumulation of hyperphosphorylated NFH in the cell body.

Whether aberrant phosphorylation of NFH is directly involved in the etiology of neurodegenerative diseases or is merely a secondary effect of the disease process remains to be demonstrated. However, in the case of ALS there are good reasons to believe that abnormal phosphorylation of NFs may be directly involved in the etiology of this disease. ALS is a neurodegenerative disease that targets the large, NF-rich motor neurons predominantly and large sensory neurons to a lesser degree (Tsukagoshi et al., 1979; Kawamura et al., 1981). Motor neurons containing proximal axonal enlargements filled with NFs (Carpenter, 1968; Hirano et al., 1984; Manetto et al., 1988; Munoz et al., 1988; Sobue et al., 1990) and abnormal hyperphosphorylation of perikaryal NFH (Manetto et al., 1988; Sobue et al., 1990) are two hallmarks of ALS.

A comparison of transgenic mice expressing murine or human NFH also supports the notion that NFH phosphorylation is important in ALS. A four-and-one-half-fold overexpression of murine NFH in transgenic mice did not cause an overt phenotype or neuronal loss, although perikaryal accumulation of NFs and proximal axonal swelling do occur in motor neurons (Marszalek et al., 1996). On the other hand, a two-fold overexpression of human NFH resulted in motor neuron degeneration (Côté et al., 1993), due to a reduction in NF transport and eventual interference with axonal transport of other components such as actin, tubulin and mitochondria (Collard et al., 1995).

The main differences between murine and human NFH are seen in the KSP repeat domain. Murine NFH has 52 KSP repeats while human NFH has 43 (Lees et al., 1988; Julien et al., 1988). However, the majority of KSP repeats in murine NFH (42/ 52) are of the KSPXE type, which is the preferred SAPK consensus sequence in NFH (see Chapter 5), while only 5 have the consensus sequence for cdk-5 ie. KSPXK, where X is not an acidic residue (Beaudette et al., 1993). Human NFH contains 17 KSP repeats with the cdk-5 consensus sequence and only 6 KSPXE sites.

The importance of the KSP repeat domain in the normal metabolism of NFs is also supported by the discovery of a small percentage of sporadic ALS cases with mutations within this domain (Figlewicz et al., 1994). One of these patients had a 34 amino acid deletion that included 5 KSP repeats. The other 4 patients identified in this study had a

single amino acid deletion following a KSP repeat which altered the sequence context from a KSPXK to a KSPXE motif. It is therefore possible that human NFH causes neurodegeneration in mouse neurons due to inappropriate phosphorylation in the axonal entry zone, which could impair NF transport. The mechanism underlying the axonal transport of NFs remains unsettled, although it is likely that NFH tail domain phosphorylation through its regulation of NFH/MT interactions is important in the transport of NFs from the cell body to the axon (see section 1.3.6). Premature phosphorylation of perikaryal NFH due to SAPK activation may cause an impairment in axonal transport of NFs similar to that seen in transgenic mice overexpressing human NFH (Collard et al., 1995).

Since the phosphorylation of perikaryal NFH is reversible (see Chapter 6), blockage of axonal transport resulting from aberrant phosphorylation of NFH might occur through the exposure of neurons to mild chronic stress or to relatively prolonged, cyclical periods of stress, perhaps coupled with a reduction in relevant phosphatase activity.

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