The effect of protein phosphatase inhibitors on neurofilament assembly

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Abstract

Treatment of dissociated cultures of rat dorsal root ganglia with 1 μM okadaic acid (OA), a serine/threonine protein phosphatase inhibitor, caused a decrease in the electrophoretic mobilities of all three neurofilament (NF) subunits, indicating an increase in their phosphorylation levels. In addition OA treatment led to the asynchronous solubilization of the subunits in Triton X-100 (Triton). This fragmentation corresponded with striking changes in the immunofluorescent staining pattern of NFs. Short-term OA treatment was fully reversible within 10 hrs. upon removal of the inhibitor from the culture medium.

Based on the response to varying concentrations of both OA and calyculin A, another serine/threonine protein phosphatase inhibitor, fragmentation of NFs was inferred to be due to the inhibition of protein phosphatase-2A activity while the reduction in electrophoretic mobility of subunits was due to the additional inhibition of protein phosphatase-1. The OA-induced phosphate moieties on the low molecular weight NF subunit (NF-L) were localized, by chemical cleavage analysis, to the amino-terminal head domain and were removed *in vitro* by the catalytic subunit of protein phosphatase-2A but not by the catalytic subunit of protein phosphatase-1.

Two-dimensional phosphopeptide mapping of the mid-sized NF subunit (NF-M) revealed that the OA-induced phosphate moieties on this subunit were also in the amino-terminal head domain. The Triton-soluble NF fragments were found, by immunoprecipitation under non-denaturing conditions, to be heteromers composed of NF-L/NF-M and NF-L/high molecular weight NF subunit. Similar heteromers were found in the small, naturally occurring Triton-soluble NF fraction from dorsal root ganglion cultures indicating that OA treatment amplifies a natural, dynamic process involving NF assembly/disassembly.

Résumé

L'acide okadaïque (AO) et la calyculine A sont des inhibiteurs de phosphatase de protéines qui agissent sur la sérine et la thréonine. Lorsque l'on utilise l'AO (1 μM) sur des cultures dissociées de ganglions dorsaux chez le rat, la mobilité électrophorétique des trois sous-unités des neurofilaments (NFs) diminue, indiquant une augmentation de leur état de phosphorylation. e plus, le traitement à l'AO solubilise les sous-unités dans le Triton X-100 (Triton) de manière non synchronisée. Cette fragmentation s'accompagne de changements très évidents dans le patron de marquage des NFs par immunofluorescence. Le traitement à l'AO à court terme était complètement réversible dans les dix heures suivant le retrait de l'inhibiteur du milieu de culture.

En se basant sur l'effet de l'AO et de la calyculine A à des concentrations différentes, la fragmentation des NFs a été attribuée à l'inhibition de la phosphatase 2A (PP2A) tandis que la diminution de la mobilité électrophorétique était attribuée à l'inhibition additionnelle de la phosphatase 1 (PP1). Suite au traitement à l'AO et à un clivage chimique, les molécules de phosphate liées à la sous-unité de faible poids moléculaire (NFL) étaient situées sur le terminal nitrogéneux de la protéine et pouvaient être enlevées in vitro à l'aide de la sous-unité catalytique de PP2A plutôt qu'avec PP1.

Le profil bidimensionel de phosphopeptides a révélé que les molécules de phosphate liées à la sous-unité de poids moléculaire moyen (NFM) suite au traitement à l'AO étaient aussi situé sur le terminal nitrogéneux. L'immunoprécipitation sous conditions non-dénaturante des NFs solubles dans le Triton a démontré que ceux-ci étaient composés d'hétéropolymères de NFL/NFM et NFL/NFH (sous unités de haut poids moléculaire). De tels hétéropolymères ont aussi été trouvés dans la fraction de NFs soluble dans le Triton que l'on trouve naturellement dans les cultures de ganglions dorsaux indiquant ainsi que le traitement à l'AO amplifie un processus dynamique naturel lié à l'assemblage et au désassemblage des NFs.

Preface

The work presented in chapters 2, 3 and 4 are entirely my own. Eric Athlan established the dorsal root ganglion culture conditions for the laboratory. Sylvia Levine performed the dissection of the ganglia and frequently dissociated and plated the cells.

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In accordance with the regulations described in the 1993 "Guidelines Concerning Thesis Preparation" booklet of the Faculty of Graduate Studies and Research and approved by the Department of Biochemistry, papers already published or submitted for publication have been incorporated into the thesis. References have been included at the end of each chapter.

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Her beautiful smile could melt the coldest Montreal winter in an instant and her sweet laughter served as a refreshing distraction in a summer full of reading and typing. It is to these two women that I dedicate this work with all of my heart.

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Abbreviations

clA	calyculin A
DARPP-32	Dopamine and cAMP-regulated phosphoprotein-32 kilodaltons
Da	Dalton
DRG	Dorsal root ganglion
EGTA	Ethyleneglycol-bis-(ß-aminoethylether)N,N'-tetraacetic acid
GFAP	Glial fibrillary acidic protein
GSK-3	Glycogen synthase kinase-3
I1	Inhibitor-1
I2	Inhibitor-2 or modulator
IF	Intermediate filament
kDa	Kilodalton
MAP	Microtubule associated protein
MF	Microfilament
MT	Microtubule
NCS	N-chlorosuccinimide
NIPP-1	Nuclear inhibitor of protein phosphatase type-1
NF	Neurofilament
NF-L	Low molecular wight neurofilament subunit
NF-M	Middle molecular wight neurofilament subunit
NF-H	High molecular wight neurofilament subunit
NMDA	N-methyl-D-aspartate
NTCB	2-Nitro-5-thiocyanobenzoic acid
OA	Okadaic acid
PAGE	Polyacrylamide gel electrophoresis
PCS	Polycation stimulated protein phosphatase
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride

PP-1	Protein phosphatase type-1
PP-2A	Protein phosphatase type-2A
PP-2B	Protein phosphatase type-2B
PP-2C	Protein phosphatase type-2C
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
Tris	Tris-(hydroxyethyl)-amino methane
Triton	Triton X-100

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Chapter 1
INTRODUCTION

1.1 The cytoskeleton

The term cytoskeleton is misleading as it implies a static, unchanging structure. In fact the cytoskeleton is known to be composed of many dynamic proteins capable of quickly responding to an everchanging external environment. The major filamentous components of the cytoskeleton are the actin microfilaments (MFs), microtubules (MTs) composed of tubulin subunits containing α- and β-tubulin and intermediate filaments (IFs), which are expressed in a tissue specific fashion. In addition to these components there is a plethora of associated proteins, some well defined others not. The following sections will describe the structure and function of the proteins which make up filamentous elements of the cytoskeleton only briefly touching upon the wealth of information on the associated proteins.

1.1.1 Microfilaments

MFs are composed of a 43,000 Da subunit referred to as globular (G)-actin (Korn, 1982). These G-actin subunits assemble into 4-6 nm wide filaments referred to as filamentous (F)-actin or MFs. The decoration of MFs by heavy meromyosin in an arrowhead pattern (Huxley, 1963) has led to the designation of the two ends of the MF as the pointed, or minus, end and the barbed, or plus, end. The rate of monomer addition at the barbed end is greater than at the pointed end.

Actin was first isolated from muscle cells (Straub, 1942) from which it was extensively studied. It was therefore assumed that its role was limited to muscle contraction. However a more general role of MFs was assumed when actin was subsequently isolated from non-muscle tissue (Hatano and Oosawa, 1966). In higher mammals six actin genes are expressed (Vandekerchove and Weber, 1984) including α-actin in muscle cells and β- and γ-actin in non-muscle cells. The ratio of β- to γ-actin in nervous tissue varies from 1:1 to 2:1 and decreases postnatally (Choo and Bray, 1978; Flanagan and Lin, 1979; Pardee and Bamburg, 1979; Otey *et al*, 1987). The need for the various isoforms of actin is unclear.

Actin has been shown to undergo a variety of post-translational

modifications including methylation (Saborio and Palmer, 1981) and removal of the amino-terminal Met with subsequent acetylation of the resulting Asp residue (Solomon and Rubenstein, 1985). ADP-ribosylation of actin increases the G-actin concentration which leads to MF disassembly (Reuner *et al*, 1987). Actin can also be phosphorylated (Dunkley and Robinson, 1983) but the significance of this form of modification is not known.

Actin binding proteins are numerous and regulate various properties of MFs or the subunits. Several proteins including profilin, depactin and vitamin-D binding protein bind preferentially to G-actin and prevent MF elongation (Stossel *et al*, 1985). A 19,000 Da protein isolated from porcine brain called *actin depolymerizing factor* binds G-actin and also severs MFs (Bamburg *et al*, 1980; Giuliano *et al*, 1988). In addition villin and gelsolin have also been shown to sever MFs as well as bind to the barbed end of MFs thus preventing further addition of G-actin (Stossel *et al*, 1985). The actions of the latter two actin binding proteins seem to be regulated by the calcium ion concentration (reviewed in Bamburg and Bernstein, 1991). Proteins which crosslink MFs, such as α-actinin and spectrin, also interact with other cytoskeletal proteins, such as vinculin, talin and ankyrin which help link the MF network to the plasma membrane allowing for cell shape regulation and response to mechanical changes (Stossel *et al*, 1985; Lazarides and Woods, 1989). The abundance of actin binding proteins illustrates the complexity of this cytoskeletal component.

1.1.2 Microtubules

MTs are composed of tubulin, a heterodimeric subunit containing two closely related polypeptides referred to as α- and β-tubulin. The tubulin subunits combine to form a tubule which contains 13 laterally associated protofilaments (Ledbetter and Porter, 1964; Tilney *et al*, 1973) and is ~20-25 nm in diameter. Both α- and β-tubulin bind 1 mole of GTP although hydrolysis of the nucleotide only occurs on the β subunit. Similar to MFs, MTs are also polarized with a

growing (plus) end and a non-growing (minus) end. Since GTP hydrolysis lags behind polymer formation (Carlier and Pantaloni, 1981) it has been suggested that a GTP cap consisting of GTP-containing tubulin subunits is present on the growing ends of MTs and only MTs with such caps are capable of incorporating tubulin subunits (Hill and Carlier, 1983; Mitchison and Kirschner, 1984).

All mammalian species possess multiple genes encoding the α- and β-tubulin polypeptides (Cleveland *et al*, 1980). Six classes of both α- (Lewis *et al*, 1985; Villasante *et al*, 1986; Hecht *et al*, 1988) and β-tubulin (Lee *et al*, 1983,1984; Hall *et al*, 1983; Lewis *et al*, 1985; Wang *et al*, 1986; Sullivan and Cleveland, 1986) have been identified. The differences between the isoforms are not clearly understood although several biochemical differences have been reported (Ludeña *et al*, 1985; Banerjee and Ludeña, 1987; Joshi *et al*, 1987) and the cell seems to be capable of distinguishing which isoform to polymerize (Joshi and Cleveland, 1988).

Post-translational modifications of tubulin include tyrosinylation-detyrosinylation of the α subunit (Barra *et al*, 1973). Removal of the carboxy-terminal tyrosine residue exposes a glutamic acid residue. Developement of antibodies specific for modified (Glu) or unmodified (Tyr) tubulin (Kilmartin *et al*, 1982; Wehland *et al*, 1984) have shown that high Glu-tubulin MTs are more stable than high Tyr-tubulin ones (Gundersen and Bulinski, 1986; Sherwin *et al*, 1987; Wehland and Weber, 1987; Kreis, 1987; Schulze *et al*, 1987). Acetylation of lysine-ϵ-amino groups in tubulin (L'Hernault and Rosenbaum, 1983,1985a) similarly contributes to MT stabilization (L'Hernault and Rosenbaum, 1985b; reviewed in Sullivan, 1988). β-tubulin has been shown to undergo phosphorylation (Gard and Kirschner, 1985) however the physiological significance of this modification is unknown. Phosphorylation seems to occur *in vivo* since isoelectric focussing has revealed up to 12 isoforms of β-tubulin which arise from the six isotypes (Field *et al*, 1984).

Many proteins are found associated with MTs prepared from brain including the family of microtubule associated proteins (MAP) 1 and 2 (MAP1A, MAP1B,

MAP1C, MAP2a, MAP2b and MAP2c) as well as MAP3, MAP4, MAP5 and the protein family referred to as tau (reviewed in Matus, 1988, Burgoyne, 1991). The MAPs are very large proteins with molecular sizes ranging from 180,000-350,000 Da while the tau family comprise proteins of 55,000-62,000 Da and 110,000 Da (Cleveland *et al*, 1979; Georgieff *et al*, 1991). Within the neuron MAP1A and MAP1B are enriched in the dendrites while MAP2a and MAP2b are found in the dendrites and cell bodies. MAP2c, MAP3 and tau are found in the axons (Bernhardt and Matus, 1984; De Camilli *et al*, 1984; Huber and Matus, 1984; Binder *et al*, 1985; Huber *et al*, 1985).

The expression of individual MAPs is developmentally regulated (see Matus, 1988, figure 2). All of these proteins are phosphorylated and their phosphorylation states alter their MT binding capabilities (Murthy and Flavin, 1983). MAPs serve to link adjacent MTs (Kanai *et al*, 1989) thus stabilizing the MT network (Gelfand and Bershadsky, 1991) as well as determining spacing between adjacent MTs (Chen *et al*, 1992). In addition, MAPs link MTs to other cytoskeletal elements such as neurofilaments (NFs) (Leterrier *et al*, 1982; Heimann *et al*, 1985) and MFs (Nishida *et al*, 1981; Selden and Pollard, 1983; Sattilaro, 1986). The functions of tau and MAP1A, at least, may be similar since mice in which tau was knocked out compensate for its loss by expressing higher amounts of MAP1A (Harada *et al*, 1994).

One of the best described, and perhaps central, role of MTs in neurons is their ability to mediate transport of membrane-bound organelles (Grafstein and Forman, 1980; Koonce and Schliwa, 1985; Schnapp *et al*, 1985; Vale *et al*, 1985a). This transport is achieved in conjunction with so called motor proteins such as kinesin and dynein (Vale *et al*, 1985b; Paschel *et al*, 1987; reviewed in Allan *et al*, 1991).

1.1.3 Intermediate filaments

IFs are found in most mammalian cells and form filaments of ~10 nm

diameter (see Steinert and Roop, 1988, for review). IF subunit proteins, expressed in a tissue specific fashion (Table 1), vary in size from 40,000 Da to 200,000 Da and share common structural features. The proteins consist of a highly conserved, 310 amino acid long α-helix-rich central rod domain, flanked by an amino-terminal head domain and a carboxy-terminal tail domain with only two instances of naturally occurring, tailless IF proteins known (Bader *et al*, 1986; Merdes *et al*, 1993). These end domains vary in length and can be further subdivided, based on homology, into charged (E) subdomains, variable (V) subdomains and high homology (H) subdomains. The variable length of the two end domains gives rise to the wide variance in IF subunit sizes.

The rod domain is highly conserved between all IF proteins and conforms to a so called heptad repeat pattern in which more than 75% of the first and fourth amino acids in a group of seven are hydrophobic. In the context of an α-helix this creates a strip of hydrophobic residues allowing for the hydrophobic interaction between two subunits leading to coiled-coil dimer formation, which represents the initial stage of IF assembly. The importance of conserving the structural features of the rod domain is highlighted by the fact that mutations within this region in keratin-14 lead to the skin blistering disease epidermolysis bullosa simplex (Bonifas *et al*, 1991; Coulombe *et al*, 1991; Rothnagel *et al*, 1992). The ends of the rod domain are highly conserved (Parry, 1991) and are essential for IF formation (Chin *et al*, 1991; Hatzfeld and Weber, 1991; Letai *et al*, 1992). This high level of sequence conservation is reflected by the availability of a monoclonal antibody which recognizes all IF proteins by binding to the carboxy-terminal portion of the rod domain (Pruss *et al*, 1981).

All IF proteins have been shown to be phosphorylated *in vitro* by either protein kinase A, calcium/calmodulin dependent protein kinase and/or protein kinase C (Eriksson *et al*, 1992). In all cases studied there is a correlation between phosphorylation of the head domain and IF disassembly. In some cases, these correlations have been made *in vivo* (discussed below).

Type	Protein name	Main tissue/cell distribution
I	Acidic keratin (about 15 proteins)	Epithelia
II	Neutral-basic keratin (about 15 proteins)	Epithelia
III	Vimentin	Fibroblasts, vascular smooth muscle cells
	Desmin	Muscle cells
	Glial fibrillary acidic protein	Glial cells
	Peripherin	Neurons of the peripheral nervous system
IV	Neurofilament (three proteins)	Most neurons of the central nervous system
	α-Internexin	Some neurons of the central nervous system
V	Nuclear lamins (two types: A and B)	Nuclear lamina
VI	Nestin	Neuroepithelial stem cells

Table 1: Classification and tissue-specific expression of intermediate filament subunits Reproduced from Skalli *et al* (1992)

The role of IFs in the cell is unclear, although they are thought to be involved in enhancing the mechanical stability of cells. During mitosis both the nuclear lamins and cytoplasmic IFs disassemble and they reassemble after cell division has taken place (Ottaviano and Gerace, 1985; Jones *et al*, 1985; Dessev and Goldman, 1988; Chou *et al*, 1989; Nishizawa *et al*, 1991; Klymkowsky *et al*, 1991). Since, in some cases, newly assembled or reassembling IFs aggregate around the nucleus and grow radially from this location (Eckert *et al*, 1982; Maro *et al*, 1983; Albers and Fuchs, 1987; Vikstrom *et al*, 1989; Chin and Liem, 1989; Soifer *et al*, 1991) IFs may also transmit mechanical signals to the nucleus allowing the cell to respond to changes in its environment (Goldman *et al*, 1986). However the existence of a cell line devoid of any cytoplasmic IFs (Sarria *et al*, 1990) and of axons devoid of NFs (Yamasaki *et al*, 1991; Eyer and Peterson, 1994) call these proposed roles of IFs into question. The tissue specific expression of IF subclasses (Steinert and Roop, 1988) may imply that these proteins serve diverse functions in different cells.

1.2 Neurofilaments: Structure and function

Neurofilaments (NFs) are composed of three subunits of low, middle and high molecular weight referred to as NF-L, NF-M and NF-H, respectively. Although generally found in neuronal cells, NFs have also been found in myelinating Schwann cells (Kelly *et al*, 1992) and in adrenal chromaffin cells (Georges *et al*, 1987) probably due to the chromaffin cells' neural crest origins (Trifaró, 1982). The apparent molecular sizes of the subunits in NFs isolated from spinal cord, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) are 68,000 Da (NF-L), 145,000 Da (NF-M) and 200,000 Da (NF-H). It was later shown that these proteins migrated anomalously on SDS-gels (Kaufmann *et al*, 1984; Georges and Mushynski, 1987).

The actual molecular masses were determined following the direct sequencing of porcine NF-L (Geisler *et al*, 1985) and DNA clones from different species by several groups. NF-L was cloned from mouse (Lewis and Cowan, 1985; Julien *et al*, 1986), human (Julien *et al*, 1987), rat (Chin and Liem, 1989) and rabbit (Soppet *et al*, 1991). All sequencing studies predict polypeptides of approximately 62,000 Da which show homologies in the range of >90%. NF-M has been cloned from mouse (Levy *et al*, 1987), rat (Napolitano *et al*, 1987) and human sources (Myers *et al*, 1987). The rat and mouse proteins are 96% homologous at the amino acid level and predict polypeptides of 95,000 Da in size. The human clone encodes a slightly larger product of 102,000 Da. NF-H has been cloned from mouse (Julien *et al*, 1988), rat (Chin and Liem, 1990) and human sources (Lees *et al*, 1988). Mouse and rat NF-H show a higher degree of homology (88%) than do human and rat NF-H (72%). Both the rat and mouse clones encode polypeptides of 115,000 Da in size while the human clone encodes a polypeptide of 110,000 Da.

The availability of their amino acid sequences has now made it possible to explain the anomalous migration of all three NF subunits on SDS-PAGE. The carboxy-terminal tail domain, especially that of NF-M and NF-H, seems to be responsible for the anomalous migration of these subunits since fusion proteins

consisting of the tail domain of either NF-M or NF-H fused to the trp-E protein migrated more slowly than expected on SDS-PAGE (Harris *et al*, 1991). NF-L, NF-M and NF-H contain high levels of glutamic acid residues in their carboxy-terminal tail domains. Modification of these acidic groups by glycinamidation increased the electrophoretic mobility of NF-L, NF-M and NF-H to 60,000 Da, 123,000 Da and 148,000 Da, respectively (Georges and Mushynski, 1987). Both NF-H and NF-M contain high amounts of lysine in their tail domains and modification of these amino acids by trifluoroacetylation increased the electrophoretic mobilities of native NF-M and NF-H. These results indicate that charged amino acids contribute to the anomalous migration of NFs on SDS-PAGE. Another factor that contributes to their anomalous migration is the high amounts of phosphate moieties found in all three subunits (Julien and Mushynski, 1981). NF-L, NF-M and NF-H have been found to contain 3, 8-25 and 14-50 moles of phosphate per mole of polypeptide, respectively (Julien and Mushynski, 1982; Jones and Williams, 1982; Carden *et al*, 1985). Removal of these phosphate moieties by alkaline phosphatase increased the electrophoretic mobilities of the NF subunits (Julien and Mushynski, 1982; Georges *et al*, 1986).

While the function of NFs in neuronal cells is still unclear and may depend on the location of the phosphate moieties on each subunit (see below), it has been suggested that NFs regulate axon calibre (Lasek *et al*, 1983; Hoffman *et al*, 1984,1987). This hypothesis appears to be supported in the case of large, myelinated fibres by the recent work of Oblinger *et al* (1989), Yamasaki *et al* (1991) and Eyer and Peterson (1994). It is also thought that NFs serve a structural role in the neuron. There is evidence to suggest that NFs and MTs interact either directly (Hisanaga and Hirokawa, 1990; Miyasaka *et al*, 1993) or via MAP2 (Hirokawa *et al*, 1988) and/or tau protein (Miyata *et al*, 1986). NF-L was shown to interact *in vitro* with brain spectrin (Frappier *et al*, 1987) thus possibly linking NFs to the plasma membrane.

The importance of NFs in neuronal cell survival has been brought into

question by some recent findings. Several reports have been published on the existence of mutant Japanese quail referred to as "quiver" mutants (Mizutani *et al*, 1992; Yamasaki *et al*, 1991,1992). These quail show no differences in fertility, hatching ability and posthatching mortality as compared to non-mutant quail. Axons of the central and peripheral nervous system lack 10 nm filaments and axon calibres are noticeably reduced (Yamasaki *et al*, 1991). The quail show a mild, generalized quivering, probably due to a reduction in the conductance velocity of myelinated axons (Sakaguchi *et al*, 1993), and lack detectable amounts of NF-L (Yamasaki *et al*, 1992). It was subsequently shown that these quail contain a nonsense mutation at the codon for amino acid residue 114 in NF-L thus preventing the synthesis of an assembly-competent subunit (Ohara *et al*, 1993). Eyer and Peterson (1994) produced transgenic mice expressing an NF-H/β-galactosidase fusion protein. This fusion protein aggregated in perikaryal regions and blocked the transport of endogenous NFs into the axon. These NF-deficient axons were of a small calibre and showed no increased degeneration relative to the axons in nontransgenic littermates. These studies imply that NFs may be involved in the determination of axon calibre but are not essential for survival. However, mice and quail are small in size and the high conductance velocities characteristic of myelinated axons may not be as important in small animals as in larger species. In a similar vein Sarria *et al* (1990) have established a cell line devoid of any cytoplasmic IF, generalizing the question of the necessity for these proteins. Interestingly two recent reports have shown that overexpression of either NF-L or NF-H in transgenic mice lead to a condition resembling amyotrophic lateral sclerosis (Coté *et al*, 1993; Xu *et al*, 1993). Therefore more work is needed to determine the actual function of and necessity for NFs.

1.2.1 Neurofilament assembly

The initial stage in NF assembly involves the formation of a coiled-coil dimer by the interaction, parallel and in axial register, of the hydrophobic strips in

the α-helix-rich rod domains of two subunits (Parry *et al*, 1985; Quinlan *et al*, 1986). It remains unclear as to whether these dimers are homo- or heterodimers since NF-L can form homopolymers *in vitro* (Geisler and Weber, 1981) whereas it cannot do so *in vivo* (Ching and Liem, 1993; Lee *et al*, 1993). The next stage in assembly is generally agreed upon to involve the formation of a tetramer from two dimers although the question of whether the dimers are in axial register or staggered and parallel or antiparallel remains unresolved (Geisler *et al*, 1985; Stewart *et al*, 1989; Coulombe and Fuchs, 1990; Hisanaga *et al*, 1990). While one group reported the existence of soluble species of NF-M and NF-H in monomeric form under restricted conditions (Cohlberg *et al*, 1987) the existence of stable tetramers consisting of only NF-L has been demonstrated (Tokutake, 1989; Tokutake and Tanaka, 1989). The formation of octamers, or protofibrils, from two tetramers is the next step in assembly but the arrangement of units remains obscure. However one report suggests that the two tetramers are aligned in an antiparallel fashion (Hisanaga *et al*, 1990).

Available evidence suggests that there are four octamers, or 32 subunits, spanning the 10 nm diameter of IFs (Steven *et al*, 1983; Ip *et al*, 1985). The distribution of each of the three NF subunits within the filament is not known. NF-L is thought to form the core of the NFs for several reasons. It is the only subunit capable of self-assembling into long, 10 nm wide filaments (Geisler and Weber, 1981; Tokutake *et al*, 1984; Gardner *et al*, 1984; Hisanaga *et al*, 1990; Balin and Lee, 1991). Furthermore, immunoelectron microscopy revealed that antibodies against the rod domain of NF-L failed to label NFs implying that this region is buried within the filaments (Balin *et al*, 1991) whereas polyclonal antibodies raised against this same subunit labelled filaments uniformly (Sharp *et al*, 1982). Finally, in IF$^-$ cells NF-H and NF-M are incapable of assembling in the absence of NF-L (Ching and Liem, 1993; Lee *et al*, 1993) and in the mutant "quiver" quail, which fails to express full length NF-L, axons are devoid of NFs (Yamasaki *et al*, 1991). In fact, recent data suggest that the basic building blocks of NFs are composed of

heterooligomers consisting of NF-L in association with either NF-M or NF-H (Mulligan *et al*, 1991; Sacher,M.G., Athlan,E.S. and Mushynski,W.E., *submitted*).

Several electron and immunoelectron microscopy studies have shown that the tail domains of NF-M and NF-H project laterally from the NF axis (Hisanaga and Hirokawa, 1988; Balin *et al*, 1991; Mulligan *et al*, 1991; Gotow *et al*, 1992; Gotow and Tanaka, 1994). These results are supported by the finding that mild treatment of NFs with chymotrypsin selectively removes the tail domains of NF-M and NF-H (Julien and Mushynski, 1983; Georges and Mushynski, 1987; Chin *et al*, 1989).

The influences of the head, rod and tail domains on NF assembly have been studied both by *in vitro* reassembly and by expression of full-length and mutated subunits in heterologous systems. One study, using full length and either amino- or carboxy-terminally deleted NF-L, concluded that the amino-terminal head domain promotes the lateral association of tetramers into octamers and, eventually, 10 nm wide filaments while the carboxy-terminal tail domain assures the termination of lateral assembly at the 10 nm level (Heins *et al*, 1993). In a similar study Nakamura *et al* (1993) showed that proteolytic removal of the tail domain of NF-L led to the formation of ribbon-like structures of greater than 10 nm diameter following reassembly. The expression of amino- and carboxy-terminal mutants of mouse NF-M and NF-L in Ltk$^-$ cells showed the dispensibility of parts of these domains in NF formation (Gill *et al*, 1990; Wong and Cleveland, 1990; Chin *et al*, 1991). In addition the ability of transfected NF subunits to co-assemble with the endogenous IF network in non-neuronal cells (Chin and Liem, 1989,1990; Gill *et al*, 1990; Wong and Cleveland, 1990; Chin *et al*, 1991; Soifer *et al*, 1991; Nash and Carden, 1991) illustrates the universality of IF assembly processes.

1.2.2 Neurofilament phosphorylation and its roles

As mentioned above all three NF subunits have been shown to contain a considerable number of phosphate moieties. The location of these phosphates

seems to influence the interactions and assembly states of NFs (discussed below). Much effort, therefore, has been put into identifying the sites of phosphorylation. Julien and Mushynski (1983) showed that most, if not all, of the phosphates on rat NF-M and NF-H were localized to a proteolytic fragment encompassing their tail domains. ^{31}P-NMR analysis revealed that the phosphates in NF-H were clustered (Zimmerman and Schlaepfer, 1986) and sequence analysis of cDNA clones of both NF-M and especially NF-H revealed a preponderance of Lys-Ser-Pro repeats in their tail domains with human (Lees *et al*, 1988), mouse (Julien *et al*, 1988) and rat NF-H (Chin and Liem, 1990) containing 43, 51 and 52 such repeats, respectively. Mouse (Levy *et al*, 1987) and rat (Napolitano *et al*, 1987) NF-M contain 5 copies of this sequence while human (Myers *et al*, 1987) NF-M contains 6 tandem repeats of a thirteen amino acid sequence each containing two copies of the Lys-Ser-Pro motif. The serine residues in these triplet repeats have been shown to be the major sites of NF phosphorylation (Geisler *et al*, 1987; Lee *et al*, 1988; Xu *et al*, 1992). Sihag and Nixon (1990) also showed low levels (5-15%) of phosphate in the head domain of NF-M. NF-L, containing fewer phosphates, was shown to be phosphorylated at one major site in both the tail domain (Xu *et al*, 1990) and the head domain (Sihag and Nixon, 1991).

The phosphate moieties in NF-M and NF-H were shown to contribute to their anomalous migration on SDS-PAGE since removal of the phosphates by alkaline phosphatase increased the electrophoretic mobility of the subunits (Julien and Mushynski, 1982; Georges *et al*, 1986). Interestingly, the catalytic subunits of protein phosphatases-1, -2A, -2B and -2C were unable to elicit a similar change in electrophoretic mobility (Hisanaga *et al*, 1993) indicating perhaps that subunits associated with these phosphatases may enhance their ability to act on NF subunits.

Many investigators have tried to identify protein kinases that co-purify with NFs or phosphorylate NFs *in vitro* and *in vivo*. Several regulator-independent protein kinase activities co-purifying with NFs have been identified (Julien and Mushynski, 1981; Runge *et al*, 1981; Shecket and Lasek, 1982; Julien *et al*, 1983).

Further fractionation of the regulator-independent protein kinase preparation revealed a casein kinase-1-like activity in rat spinal cord (Julien *et al*, 1983; Toru-Delbauffe and Pierre, 1983), bovine spinal cord (Link *et al*, 1993) and squid axoplasm (Floyd *et al*, 1991). In addition a NF kinase capable of phosphorylating several other proteins (Toru-Delbauffe *et al*, 1986) and a NF-specific kinase which phosphorylates only native, highly phosphorylated NF-H (Wible *et al*, 1989) have been identified from this fraction. A NF associated kinase was purified from chicken spinal cord (Hollander and Bennett, 1992) which phosphorylated some, but not all, Lys-Ser-Pro sites on the tail domain of NF-M (Hollander *et al*, 1993).

Calcium/calmodulin-dependent protein kinase II was also found to co-purify with NFs (Vallano *et al*, 1985; Caputo *et al*, 1989; Dosemeci *et al*, 1990) and may be involved in their phosphorylation *in vivo* (Sihag and Nixon, 1989). Both cAMP-dependent protein kinase (PKA; Caputo *et al*, 1989; Dosemeci *et al*, 1990) and protein kinase C (PKC; Dosemeci *et al*, 1990) have been shown to co-purify with NFs. PKC phosphorylated NFs *in vitro* (Sihag *et al*, 1988) and may be involved in their phosphorylation *in vivo* (Georges *et al*, 1989; Grant and Aunis, 1990). *In vitro* phosphorylation of NF-M and NF-L by PKA occurs in the amino-terminal head domain (Sihag and Nixon, 1990,1991; Hisanaga *et al*, 1994) and some of these sites are phosphorylated *in vivo*. An association between the catalytic subunit of PKA and NFs was shown to be blocked by the addition of the regulatory subunit of PKA and reversal of the block was effected by the addition of cAMP (Dosemeci and Pant, 1992) indicating that the interaction of PKA with NFs may be a second messenger-regulated event. A novel 115,000 Da protein kinase was recently identified by its ability to bind to a column composed of the tail domain of NF-H expressed in bacteria (Xiao and Monteiro, 1994). An *in vivo* association between this kinase and NFs was shown by its ability to be co-immunoprecipitated with NFs.

Several protein kinases which do not co-purify with NFs have been shown to phosphorylate NFs *in vitro* including glycogen synthase kinase-3 (GSK-3; Guan

et al, 1991), two novel protein kinases from bovine brain (Roder and Ingram, 1991) and cdc2 kinase from both starfish oocytes (Hisanaga *et al*, 1991) and a mouse mammary carcinoma (Guan *et al*, 1992). Subsequently, cdc2-related kinases, now known to be cdk5, were purified from neuronal tissue (Hellmich *et al*, 1992; Lew *et al*, 1992; Kobayashi *et al*, 1993) and shown to phosphorylate the consensus sequence Ser/Thr-Pro-X-Lys (Beaudette *et al*, 1993; Hisanaga *et al*, 1993; Shetty *et al*, 1993) that is found in both NF-M and NF-H. Recently a proline-directed casein kinase activity from bovine brain was characterized which, like cdk5, showed a requirement for a proline residue on the carboxy-terminal side of the phosphorylated residue (Angelov, 1994). Its size (45,000 Da) distinguishes it from cdk5 (33,000 Da), however its ability to phosphorylate NFs was not determined.

Since all of the protein kinases discussed above seem to phosphorylate different sites on NF subunits and some, such as GSK-3 and NF-specific kinase, require prior phosphorylation of NFs, it is possible that complete phosphorylation of NFs is accomplished by the concerted effort of several protein kinases perhaps in a hierarchical fashion (Roach, 1991).

The ability of protein kinases to co-purify with NFs may be artifactual due to the highly charged nature of NF subunits. Several studies have shown that the expression of NF-H cDNA in non-neuronal cells yields an unphosphorylated protein (Chin and Liem, 1990; Nash and Carden, 1991; Soifer *et al*, 1991). On the other hand, NF-M expressed in non-neuronal cells was indeed phosphorylated on some Lys-Ser-Pro sites (Chin and Liem, 1989; Pleasure *et al*, 1990) indicating that at least some of the protein kinases acting on NFs are either active only in neurons or are neuron enriched such as cdk5.

Phosphorylated NF subunits are compartmentalized within the neuron with axonal subunits being more highly phosphorylated than their perikaryal and dendritic counterparts (Sternberger and Sternberger, 1983). However following aluminum intoxication (Bizzi and Gambetti, 1986) and in certain neurodegenerative diseases (Sternberger *et al*, 1985; Wiley *et al*, 1987; Muñoz *et*

al, 1988) perikaryal NFs are found to be in a hyperphosphorylated state. Whether this is a cause or a consequence of the pathological state is unknown.

The once obscure role of NF subunit phosphorylation is now starting to clear. Available evidence indicates that amino-terminal head domain phosphorylation of NF subunits regulates their assembly states while phosphorylation of the carboxy-terminal tail domains of NF-M and NF-H regulates the interactions of these projections with adjacent filaments and other cellular components (Fliegner and Liem, 1991; Nixon and Sihag, 1991).

Several reports have shown that phosphorylation by either PKA or PKC caused homopolymeric filaments composed of NF-L to disassemble and prevented monomers from assembling past the octamer stage (Gonda *et al*, 1990; Nakamura *et al*, 1990; Hisanaga *et al*, 1990,1994). In these studies PKA added 2-4 moles of phosphate per mole of NF-L polypeptide. The residues in NF-L that were phosphorylated by PKA were shown to reside in the amino-terminal head domain with one site at serine-55 (Sihag and Nixon, 1991) and at least one each on amino-terminal peptides comprising amino acid residues 1-14 and 37-53 (Hisanaga *et al*, 1994). One study examined the differences between native NFs and reassembled NFs in terms of their ability to disassemble following phosphorylation by PKA (Hisanaga *et al*, 1994). It was found that native NFs are relatively resistant to PKA-mediated disassembly as compared to reassembled NFs although the native NFs showed a partial fragmentation as determined by differential centrifugation. Electron microscopy showed regions of thinning along filaments which may be sites where the NFs were severed after phosphorylation. These results are interesting in that they show differences between reassembled and native NFs in addition to their previously noted differences in length (Balin *et al*, 1991). In addition the results of Hisanaga *et al* (1994) imply that reassembly *in vitro* differs from that *in vivo* in either the structural arrangement of NFs or in factors which hold the filaments together. In this respect it is noteworthy that many IF-associated proteins (IFAP) have been identified, some of which seem to link adjacent filaments (Yang

et al, 1991). These include a neuron-specific protein called NAPA-73 (Ciment *et al*, 1986). Interestingly, one of these IFAPs, which is found in BHK-21 cells, is present at crossover regions along vimentin IFs (Yang *et al*, 1991) and may aid in network stabilization. NF-M was also shown to be phosphorylated in the amino-terminal head domain by PKA both *in vivo* and *in vitro*. The effect of this head domain phosphorylation on NF-M assembly has not been determined directly since self assembly of this subunit fails to produce long, 10 nm wide filaments.

It is not clear whether head domain phosphorylation of NFs is physiologically relevant although a correlation between NF fragmentation and head domain phosphorylation of both NF-M and NF-L has recently been shown (Sacher *et al*, 1994). Since amino-terminal domain phosphorylation of other IFs correlates with their disassembly both *in vitro* and *in vivo* (Inagaki *et al*, 1987,1988,1989,1990; Geisler and Weber, 1988; Ando *et al*, 1989; Geisler *et al*, 1989; Chou *et al*, 1990,1991; Heald and McKeon, 1990; Nishizawa *et al*, 1991) it seems likely that reversible head domain phosphorylation is a general mechanism in the regulation of IF assembly/disassembly.

Whether there is a need to disassemble the IF network in a non-dividing cell such as the neuron remains an open question. In dividing cells the disassembly of IFs is correlated with mitosis (Chou *et al*, 1989) and phosphorylation is mediated by effector-independent kinases such as cdc2 kinase (Rosevear *et al*, 1990; Chou *et al*, 1991; Eriksson *et al*, 1992). In the case of NFs head domain phosphorylation is mediated by second messenger dependent kinases such as PKA and PKC (see above). The dynamic nature of NFs has been demonstrated both *in vitro* (Angelides *et al*, 1989) and *in vivo* (Sacher *et al*, 1992,1994; Okabe *et al*, 1993; reviewed in Nixon and Shea, 1992). It is therefore possible that head domain phosphorylation causes a local disruption of the NF network allowing for an exchange between NFs and a soluble, unassembled pool of oligomeric subunits. Such phosphorylated, soluble, NF-H-containing oligomers have been identified in neuroblastoma NB2a/d1 cells (Shea *et al*, 1988) as well as in Purkinje and anterior horn cells

(Gotow and Tanaka, 1994). These NFs may represent a pool of oligomers in equilibrium with NFs, as opposed to nascent polypeptides, since most phosphates seem to be added to NF-H after incorporation into the filaments (Nixon *et al*, 1989). However, the location of the phosphate moieties on these soluble NF-H oligomers was not determined and may not involve the head domain since the oligomers were assembly competent (Shea, 1994). In addition, NFs assume a less random architecture at nodes of Ranvier as compared to myelinated regions of the axon (Price *et al*, 1993). Perhaps differences in head domain phosphorylation of NF subunits throughout the axon allow for such structural changes.

Phosphorylation of the tail domain of both NF-M and NF-H seems to be effected by second messenger-independent kinases (Vallano *et al*, 1985; Wible *et al*, 1989; Hisanaga *et al*, 1991,1993; Roder and Ingram, 1991; Lew *et al*, 1992; Shetty *et al*, 1992; Hollander *et al*, 1993; Xiao and Monteiro, 1994) and may regulate interactions with surrounding structures such as MTs. Phosphorylation of the tail domain of NF-H by either cdc2 kinase (Hisanaga *et al*, 1991) or tau protein kinase II (Miyasaka *et al*, 1993) reduced its ability to bind to MTs *in vitro* and dephosphorylation of NFs restored this interaction (Hisanaga *et al*, 1993). Interestingly, tau protein kinase II was shown to be identical to cdk5 (Kobayashi *et al*, 1993).

It has been suggested that phosphorylation of the tail domains of NF-M and NF-H causes them to project laterally from the filament axis and thereby increase the distance between adjacent filaments (Carden *et al*, 1987). This notion is compatible with the suggestion that NFs determine axonal calibre (see above), although Hisanaga and Hirokawa (1989) showed that dephosphorylation of NFs by alkaline phosphatase had no effect on the appearance of the projections nor on their ability to form crossbridges. Shaw (1991) proposed that phosphorylation of the tail domains of NF-M and NF-H would influence axon calibre by causing a repulsion between these projections thereby influencing their positions but not their lengths.

Several studies examined the interactions of the tail domains of NF-M and NF-H in attempts to explain the reason for the projections and crossbridges seen between NFs. Chin *et al* (1989) showed, by removal of the tail domains of the two subunits by chymotrypsin, that they do not interact in solution with each other nor with native or dephosphorylated NFs. Gotow *et al* (1992) take issue with this and argue that the tails of NF-M and NF-H do interact to form crossbridges. This is based on the fact that reassembled filaments containing NF-L and NF-H contain the same density of crossbridges and projections as filaments reassembled from all three subunits. Since reassembled filaments containing NF-M and NF-L only form projections these investigators reasoned that in the presence of NF-H the tail domain of NF-M must form crossbridges. In addition Eyer and Leterrier (1988) show an increase in the viscosity of a suspension of NFs following phosphorylation suggesting an increased interaction between filaments. Perhaps the discrepancy between these studies is due to the use of proteolytically derived tail domains by Chin *et al* (1989) while the latter two studies used the intact subunits. A later study (Gotow and Tanaka, 1994) showed that the NF-H tail domain is highly phosphorylated when it forms crossbridges with adjacent NFs (in the axon) but is poorly phosphorylated when the filaments appear singly (in the perikaryon). Interestingly, these authors point out that axonal MTs are segregated from NFs. Perhaps phosphorylation of the tail domain of NF-H in the axon allows NF-NF interactions to take place to the exclusion of NF-MT interactions. The opposite would occur in the perikaryon since, as mentioned above, NF-MT interactions *in vitro* are increased following dephosphorylation of NFs (Hisanaga *et al*, 1993).

Finally, NF phosphorylation has been suggested to influence diverse processes such as the protection of NFs from proteolysis (Goldstein *et al*, 1987; Pant, 1988) and influencing their structure (Otvos *et al*, 1988). The ability of native and dephosphorylated NFs to bind calcium has also been reported (Lefebvre and Mushynski, 1987) but its significance is still unclear. However, Holly *et al* (1993) reported that calcium binding influences NF-M secondary structure.

1.3 Protein phosphatases

The most common method for controlling the structure and function of cellular proteins is reversible phosphorylation. The phosphorylation state of any phosphoprotein is dictated by the balance between the activities of protein kinases and protein phosphatases. For a long time it was thought that protein phosphatases comprised a relatively small group of proteins whose activity was not regulated as tightly as that of protein kinases. Research in the protein phosphatase field therefore lagged behind that of protein kinases. The recent discovery of various isozymes of serine/threonine protein phosphatase catalytic subunits, targeting subunits and associated proteins (for reviews see Cohen, 1989; Shenolikar and Nairn, 1991; Bollen and Stalmans, 1992) and the rapid expansion of protein tyrosine phosphatase families (Charbonneau and Tonks, 1992) represents an explosion of knowledge that has aided in the answering of old questions and the generation of new ones in this area of research. The following sections deal with the major serine/threonine protein phosphatases of eukaryotic cells, their classification, functions and modes of regulation. Emphasis is placed on the type-1 and type-2A phosphatases (see below) due to their apparent involvement in NF metabolism (Sacher *et al*, 1992,1994).

1.3.1 Classification and structure of protein phosphatases

The most widely accepted method for classifying protein phosphatases is that of Ingebritsen and Cohen (1983). This method tests the ability of the phosphatase to dephosphorylate the α and β subunits of phosphorylase kinase and measures its response to the thermostable protein phosphatase inhibitors 1 and 2 (I1 and I2, respectively; note that I2 is sometimes referred to as "modulator") (Huang and Glinsmann, 1976). Protein phosphatases of the type-1 (PP-1) class preferentially dephosphorylate the β-subunit of phosphorylase kinase and are inhibited by I1 and I2 whereas those of the type-2 (PP-2) class are resistant to inhibition by I1 and I2 and preferentially dephosphorylate the α-subunit of phosphorylase kinase. The PP-2 class can be further subdivided on the basis of

requirement for divalent cations. The *A* subclass (PP-2A) requires no cations for activity, the *B* subclass (PP-2B) requires calcium and the *C* subclass (PP-2C) requires manganese. Furthermore, the non-phorbol tumour promoter, okadaic acid (OA), has been shown to inhibit PP-2A (K_i 0.2 nM) with a higher potency than PP-1 (K_i 20 nM) (Bialojan and Takai, 1988) thus allowing a further means of distinguishing between these two enzymes. An inactive species consisting of PP-1 and I2 is occasionally referred to as the *ATP-magnesium* dependent protein phosphatase (Merlevede and Riley, 1966) or F_c (Goris *et al*, 1979), due to its requirement for these two substances (discussed below). Since polycations such as poly-lysine and protamine stimulate PP-2A (Pelech and Cohen, 1985) this subclass is often referred to as the *polycation stimulated* protein phosphatase or PCS. The fact that PP-2B requires calcium and is localized predominantly in neural tissue (Klee and Cohen, 1988) led to the neuronal species of this phosphatase being named *calcineurin*.

A structural comparison of protein phosphatases reveals a high degree of conservation of catalytic subunits throughout evolution indicating that the enzymes play an essential role in cell viability. For example, the catalytic subunits of PP-1 ($PP\text{-}1_c$) from fungi, fruit flies and humans share over 86% sequence identity while PP-2A catalytic subunits ($PP\text{-}2A_c$) from fruit flies and mammals share 93% sequence identity (Shenolikar and Nairn, 1991). $PP\text{-}1_c$ and $PP\text{-}2A_c$ share 41% sequence identity (Berndt *et al*, 1987) which may explain the cross-reactivity of certain monoclonal antibodies with both proteins (Brautigan *et al*, 1986). In addition, $PP\text{-}1_c$, $PP\text{-}2A_c$ and the catalytic subunit of PP-2B ($PP\text{-}2B_c$) share six highly conserved regions which may form part of the catalytic domain. These enzymes are thought to have evolved from a common lineage. $PP\text{-}2B_c$ may haves arisen as the result of fusion with a gene encoding a calmodulin binding protein (Ito *et al*, 1989). The catalytic subunit of PP-2C shows little homology with the aforementioned enzymes and is thought to have diverged early in evolution. Recently five distinct protein phosphatases termed PP-V, PP-X, PP-Y, PP-Z and $PP\text{-}2B_\gamma$ were cloned

from a *drosophila* cDNA library (Cohen *et al*, 1990). PP-X and PP-V are more similar to PP-2A (57-69% homology) than to PP-1 (45-49% homology) while PP-Y and PP-Z are more similar to PP-1 (66-68% homology). PP-$2B_w$ is a calcium/calmodulin dependent protein phosphatase with 54% homology to PP-2B (da Cruz e Silva and Cohen, 1989).

1.3.2 Protein phosphatase-1: Structure and properties

PP-1_c has a molecular mass of 37,000 Da. Two isoforms of PP-1_c termed PP-$1_c\alpha$ and PP-$1_c\beta$ have been cloned from rabbit skeletal muscle (Cohen, 1989; Barker *et al*, 1994). The *dis*2 gene of the fission yeast shows strong homology with rabbit skeletal muscle PP-$1_c\alpha$. Screening of a mouse fetal brain cDNA library with a *dis*2 probe yielded two distinct clones, termed *dis2m1* and *dis2m2* which are 90% identical (Ohkura *et al*, 1989). These cDNAs were subsequently found in both rat liver and testis and were called PP-$1_c\gamma 1$ and PP-$1_c\delta$, respectively (Sasaki *et al*, 1990). A fourth clone, called PP-$1_c\gamma 2$ and abundant in rat testis, was also identified and found to be an alternatively spliced form of PP-$1_c\gamma 1$. The human forms of PP-$1_c\alpha$, PP-$1_c\beta$ and PP-$1_c\gamma$ have all been cloned (Barker *et al*, 1990,1993,1994). All of the isoforms of PP-1_c are sensitive to inhibition by I2 and okadaic acid (see below) (Zhang *et al*, 1993).

PP-1_c is found associated with several regulatory components which determine subcellular localization as well as activity against various substrates. In skeletal muscle, liver, brain and kidney more than 70% of the PP-1 activity was sedimented by centrifugation at 100,000xg (Cohen, 1989). In rabbit skeletal muscle 30-60% of the PP-1 activity was associated with glycogen particles (Ingebristen *et al*, 1983; Hubbard *et al*, 1990) where PP-1_c was found in a 1:1 complex with a 161,000 Da protein called the *glycogen binding subunit* or G subunit (Strålfors *et al*, 1985). The G subunit binds both glycogen and PP-1_c with high affinity (k_d of 8 nM and 4 nM, respectively) (Hubbard and Cohen, 1989). The G subunit is found in skeletal muscle, heart and diaphragm but not in kidney, liver, lung or brain (Bollen

and Stalmans, 1992). This indicates that these other tissues contain similar proteins which link PP-1 activity with the particulate fraction. Interestingly, Wera *et al* (1991) purified a 161,000 Da protein thought to be the rat liver homologue of the G subunit.

Another form of PP-1 termed PP-1M can be found associated with skeletal muscle myofibrils. This complex, with a molecular mass of 110,000 Da, binds specifically to actomyosin (Chisholm and Cohen, 1988). The binding of the M subunit to actomyosin is weaker than that of the G subunit to glycogen since 300 mM NaCl will release the former but not the latter.

The most extensively studied form of PP-1, first identified in bovine adrenal cortex (Merlevede and Riley, 1966), has been termed the ATP-magnesium dependent form, since preincubation with these additives is required to generate phosphatase activity. It is also referred to as F_c since an activating factor, termed F_A, was found to be required for activating the phosphatase (Goris *et al*, 1979). The activating factor was found to phosphorylate the enzyme glycogen synthase (Vandenheede *et al*, 1980) and was later shown to be glycogen synthase kinase-3 (GSK-3) (Hemmings *et al*, 1981). It was subsequently shown that F_c was composed of a 1:1 complex between PP-1_c and I2 (Yang *et al*, 1981,1983) and phosphorylation of I2 led to the activation of PP-1_c (discussed in detail below) (Ballou *et al*, 1983; Jurgensen *et al*, 1984).

PP-1 activity has also been identified in the nucleus of rat liver cells (Kuret *et al*, 1986), HeLa cells (Friedman, 1986) and *Xenopus* oocytes (Jessus *et al*, 1989). The concentration of enzyme in liver nuclei is 5-fold higher than extranuclear levels (Kuret *et al*, 1986). Between 40% and 80% of the nuclear PP-1 (PP-1N) is particulate which may be explained by its apparent association with chromosomes (Fernandez *et al*, 1992). PP-1_c in nuclei is complexed with a 16,000-18,000 Da polypeptide called *Nuclear Inhibitor of PP-1*, or NIPP-1.

1.3.2.1 Regulation of protein phosphatase-1

It is now well established that extranuclear PP-1 activity is regulated by second messengers. As mentioned above, skeletal muscle PP-1_c can be found complexed to the G subunit thus binding the enzyme to glycogen. This form of the enzyme, PP-1G, is active. However, several phosphorylation sites exist on the G subunit which, when phosphorylated, cause the release of PP-1_c. Phosphorylation by PKA (Caudwell *et al*, 1986; Hubbard and Cohen, 1989) causes PP-1_c to dissociate from the G subunit, which remains bound to glycogen (Hubbard and Cohen, 1989). Phosphorylation by insulin-stimulated protein kinase does not lead to a similar dissociation of the catalytic subunit but does increase PP-1G activity toward certain substrates (Dent *et al*, 1990). GSK-3 phosphorylates two different sites *in vitro* (Fiol *et al*, 1988; Dent *et al*, 1989) one of which is phosphorylated *in vivo* as well. In addition casein kinase-2 acts on the G subunit *in vitro* (Bollen and Stalmans, 1992).

PP-1_c released into the cytosol by PKA phosphorylation of the G subunit can be complexed to and inhibited by I1 after the latter is phosphorylated by the same protein kinase (discussed below) (Foulkes *et al*, 1983). Thus the dual action of PKA on the G subunit and I1 results in the inactivation of PP-1_c. Injection of adrenalin into rabbits causes a reduction in PP-1 activity by this mechanism (Hiraga and Cohen, 1986). The injection of insulin leads to an increase in G subunit phosphorylation by insulin-stimulated protein kinase which causes an increased phosphatase activity toward glycogen synthase and phosphorylase kinase (Dent *et al*, 1990). There is also a decrease in I1 phosphorylation resulting in a further increase in PP-1 activity (Chang and Huang, 1980).

Regulation of hepatic PP-1G differs from that of skeletal muscle PP-1G since dissociation of liver PP-1_c from the glycogen binding subunit does not occur in the presence of PKA nor has it been shown that the subunit can even be phosphorylated (Wera *et al*, 1991). In addition rat and mouse liver are devoid of I1 and its brain homologue DARPP-32 (discussed below) (Huang *et al*, 1977;

MacDougall *et al*, 1989; Elbrecht *et al*, 1990). Instead the phosphorylation states of phosphorylase and phosphorylase kinase regulate the activity of hepatic PP-1G (Alemany and Cohen, 1986). Furthermore, agents which increase intracellular calcium levels, such as vasopressin and angiotensin, act to increase phosphorylase a levels thus inhibiting PP-1G (Cohen, 1989).

Beullens *et al* (1992) showed that the activity of PP-1N is controlled by its association with NIPP-1 and that an inactive complex of NIPP-1:PP-1_c could be isolated from bovine thymus nuclei (Beullens *et al*, 1993). This inactive species could be activated up to 4-fold by phosphorylation with either PKA or casein kinase-2 (Beullens *et al*, 1993; Van Eynde *et al*, 1994) with an additive 8-fold activation after treatment with both protein kinases (Van Eynde *et al*, 1994). It was not determined whether phosphorylation caused a dissociation of PP-1_c from NIPP-1 or merely an activation of the complex as is seen with the ATP-magnesium dependent form of PP-1 (see below).

Cytoplasmic inhibitory proteins of PP-1_c exist as well. Two of these, the thermostable and acid stable inhibitors I1 and the *dopamine and cAMP regulated phosphoprotein* (DARPP-32) are closely related (Shenolikar and Nairn, 1991) and inhibit PP-1_c in a similar fashion. I1 and DARPP-32 have molecular masses of 18,700 Da and 22,600 Da respectively (Aitken *et al*, 1982; Williams *et al*, 1986). While mouse and rat liver contain neither I1 nor DARPP-32 (Huang *et al*, 1977; MacDougall *et al*, 1989; Elbrecht *et al*, 1990) I1 is found in mammalian skeletal muscle, heart, kidney, uterus and adipose tissue (Huang and Glinsmann, 1976; Huang *et al*, 1977; MacDougall *et al*, 1989). DARPP-32 is present in brain regions with dopaminergic neurons, brown adipose tissue, adrenal medulla and pineal gland (Hemmings and Greengard, 1986; Meister *et al*, 1988). Since the concentration of I1 is 3-4 times higher than that of PP-1_c (Nimmo and Cohen, 1978; Ingebritsen *et al*, 1983) phosphatase activity can be quickly regulated. Both I1 and DARPP-32 are inhibitory towards PP-1_c only after phosphorylation by PKA (Hemmings *et al*, 1984a,b). Additionally, DARPP-32 can be phosphorylated by

casein kinase-2, which does not increase its potency as an inhibitor of PP-1_c but does promote phosphorylation by PKA (Girault *et al*, 1989). The inhibitory domain of both DARPP-32 and I1 was determined to reside in their extreme amino-terminal portions (Aitken and Cohen, 1982; Hemmings *et al*, 1990).

Dephosphorylation of both I1 and DARPP-32 is catalyzed by PP-2A and PP-2B (Hemmings *et al*, 1984a,b,c; King *et al*, 1984). Activation of N-methyl-D-aspartate (NMDA) receptors counteracts forskolin-induced DARPP-32 phosphorylation. NMDA increases intracellular calcium which may activate PP-2B allowing it to dephosphorylate DARPP-32 (Halpain *et al*,1990). This supports a model of antagonism between calcium and cAMP (see Cohen, 1989, figure 1). DARPP-32 phosphorylated by casein kinase-2 is a poor substrate for PP-2B but is dephosphorylated by both PP-1 and PP-2A suggesting a complex regulation of its function (Cohen, 1989). Finally it is noteworthy that PP-1 found in the particulate fraction of rat brain has a significantly lower specific activity compared to the soluble form (Sim *et al*, 1994). The basis for this observation is unclear since brain specific regulatory subunits of PP-1 (other than DARPP-32 which is soluble) are not known.

Another heat and acid stable inhibitor of PP-1_c was isolated together with I1 and is called I2 or modulator (Huang and Glinsmann, 1976). I2 has been found in all tissues examined including those in which I1 and DARPP-32 are lacking. Like I1 and DARPP-32, I2 is also phosphorylated although phosphorylation, by GSK-3, abolishes the protein's inhibitory effect (Vandenheede *et al*, 1983). I2 is also phosphorylated *in vitro* by casein kinases-1 and 2 as well as by PKA (DePaoli-Roach, 1984; Holmes *et al*, 1986; Agostinis *et al*, 1987). Phosphorylation by PKA has no effect on the F_c complex and is probably not physiologically relevant. The sites phosphorylated by casein kinase-2 also occur *in vivo* and, while not deinhibitory by themselves, increase the rate of phosphorylation by GSK-3 (DePaoli-Roach, 1984).

Although initially it was thought that I2 phosphorylation by GSK-3 caused

it to dissociate from PP-1_c this is now known to be false. I2 remains bound throughout the entire activation process. Phosphorylation of I2 by GSK-3 causes a conformational change in I2 exposing a metal ion binding site on PP-1_c (Li *et al*, 1985; Price and Li, 1985; Price *et al*, 1986). The subsequent binding of magnesium to PP-1_c causes it to dephosphorylate the associated I2 polypeptide resulting in the formation of an active complex (Li *et al*, 1985; Price and Li, 1985). Subsequent loss of magnesium allows the complex to revert back to the ATP-magnesium dependent form (Abeele *et al*, 1987). Recently Villa-Moruzzi (1992) showed that cdc2 kinase was capable of replacing GSK-3 in the activation of F_c.

Recently the role of I2 was proposed to be that of a chaperone (Alessi *et al*, 1993) assisting in the proper folding of PP-1_c. The basis for this suggestion was that three recombinant PP-1_c isoforms (PP-$1_c\alpha$, -β and -γ1), expressed in bacteria, showed several striking differences to PP-1_c from rabbit skeletal muscle. Namely, the bacterially expressed isoforms were less sensitive to inhibition by okadaic acid, I1 and microcystin-LR and showed increased activity in dephosphorylating histone H1 than did muscle PP-1_c. Association of these isoforms with I2 inhibited their activity and subsequent phosphorylation of I2 by GSK-3 (see above) altered their properties to those of skeletal muscle PP-1_c. Furthermore, I2 was required to restore the activity of PP-$1_c\gamma$1 which was denatured in 6 M guanidine. The G subunit was capable of displacing phospho-I2, but not unphosphorylated I2, from PP-1_c. These results led these authors to conclude that the formation of an I2:PP-1_c complex may be an intermediate in the production of all forms of active PP-1_c. Subsequently, PP-$1_c\gamma$2 was found to be associated with a 78,000 Da protein and a 55,000 Da protein. The larger protein was identical to the 78,000 Da glucose-regulated protein, a member of the 70,000 Da heat shock protein (HSP-70) family (Chun *et al*, 1994) and a putative molecular chaperone. It was suggested that the 78,000 Da protein may serve to target PP-$1_c\gamma$2 to the nucleus since this phosphatase has no nuclear localization signal (Chun *et al*, 1994). However, it may serve a function not unlike that described above for I2 since it was also shown *in*

vitro that purified HSP-70 activates PP-1 in rabbit reticulocyte lysates (Mivechi *et al*, 1993). Recently Bollen *et al* (1994) purified a spontaneously active PP-1_c:I2 complex which could not be further activated by GSK-3. Therefore, while more work is needed to clarify the role of I2, it is clear that this protein has a role more complex than as a PP-1 inhibitor.

1.3.2.2 Physiological roles of protein phosphatase-1

PP-1 activity was shown to be involved in maintaining the structural organization of MTs, IFs (Eriksson *et al*, 1992) and MFs (Fernandez *et al*, 1990), thereby influencing processes such as organelle movement and phagocytosis.

In skeletal and cardiac muscle PP-1M has been implicated in muscle contraction because of its role in controlling the state of myosin-p light chain phosphorylation (Hoar *et al*, 1985).

The involvement of PP-1 in protein synthesis has been suggested since it is the major S6 phosphatase in mouse 3T3 cells (Olivier *et al*, 1988). Also, addition of I2 to rabbit reticulocyte lysates increases the extent of phosphorylation of the α-subunit of initiation factor eIF-2 and inhibits peptide chain initiation (Ernst *et al*, 1982).

Protein phosphatases in fission yeast and *Aspergillus nidulans* with high homology to PP-$1_c\alpha$ are involved in chromosomal disjoining during mitosis (Ohkura *et al*, 1989; Doonan and Morris, 1989). In *Drosophila* the loss of one isoform of PP-1_c, representing 80% of PP-1 activity, is lethal and larvae show defects in mitosis (Dombrádi *et al*, 1990). A testis-specific isoform of PP-1_c, PP-$1_c\gamma 2$, was found to localize to the nucleus of meiotic spermatocytes (immature spematozoa) and spermatids (mature spermatozoa) (Shima *et al*, 1993) suggesting that it plays a role in meiosis.

Several other roles for PP-1 have been suggested including calcium transport (Klumpp *et al*, 1990; MacDougall *et al*, 1991) and, as described above, glycogen metabolism. In addition, PP-1 activity was shown to be significantly lower in

Alzheimer's diseased brain than in control brain (Gong *et al*, 1993) and the phosphatase was shown to dephosphorylate 4 sites on the microtubule-associated protein tau which are abnormally phosphorylated in Alzheimer disease brain (Gong *et al*, 1994).

1.3.3 Protein phosphatase-2A: Structure and properties

As mentioned above protein phosphatases of the type 2 class can be subdivided based on their requirements for divalent cations. PP-2A shows no requirement for divalent cations (Ingebritsen and Cohen, 1983). Since its activity is stimulated by polycations such as poly-lysine, histone H1 and protamine (Waelkens *et al*, 1987), it is also referred to as *polycation stimulated protein phosphatase* or PCS. PP-2A consists of a catalytic subunit with one or more associated subunits which presumably dictate localization and substrate specificity. Prior to discussing the various holoenzyme forms of the phosphatase the catalytic subunit ($PP\text{-}2A_c$) will briefly be described.

Two isoforms of $PP\text{-}2A_c$, termed $PP\text{-}2A_c\alpha$ and $PP\text{-}2A_c\beta$, have been cloned from rabbit skeletal muscle and porcine kidney (da Cruz e Silva *et al*, 1987; da Cruz e Silva and Cohen, 1987; Stone *et al*, 1987). The 35,600 Da proteins show 97% amino acid identity. Most of the differences are located in the amino-terminal 40 residues and at the nucleotide level the 3' noncoding regions are completely different. $PP\text{-}2A_c\alpha$ from rabbit skeletal muscle and porcine kidney were identical whereas $PP\text{-}2A_c\beta$ differed at a single amino acid. $PP\text{-}2A_c\alpha$ has also been cloned from bovine adrenal cortex (Green *et al*, 1987), rat liver (Kitagawa *et al*, 1988) and human liver (Arino *et al*, 1988). $PP\text{-}2A_c\beta$ has been cloned from rabbit liver (da Cruz e Silva *et al*, 1988) and human liver (Arino *et al*, 1988). All isoforms of $PP\text{-}2A_c$ are almost completely conserved at the amino acid level indicating an essential role for this phosphatase.

Three forms of the PP-2A holoenzyme can be purified from rabbit skeletal muscle referred to as $PP\text{-}2A_0$, $PP\text{-}2A_1$ and $PP\text{-}2A_2$ based on their elution from

DEAE-cellulose (Ingebritsen *et al*, 1983). All three forms contain a catalytic (C) and a 60,000-65,000 Da (A) subunit but PP-$2A_0$ and PP-$2A_1$ each contained one additional subunit called B' (54,000 Da) and B (55,000 Da), respectively. The A and C subunits are tightly bound to one another (Kobayashi *et al*, 1975; Imazu *et al*, 1978; Imazu *et al*, 1981). Various other forms of high molecular weight enzymes have been purified from mammalian tissue and shrimp (Table 2; Waelkens *et al*, 1987; Mumby *et al*, 1987; Usui *et al*, 1988; Hermann *et al*, 1988). Generally speaking the core of these PP-2A phosphatases consists of the catalytic subunit and a 65,000 Da subunit which can associate with either 54,000 Da, 55,000 Da, 72,000 Da or 74,000 Da subunits (Hendrix *et al*, 1993).

The core structure of PP-$2A_1$ consists of the catalytic subunit, the 60,000 Da subunit and the B subunit (55,000 Da). Mayer *et al* (1991) cloned the B subunit from human and rabbit fetal brain and discovered two isoforms called PR55α and PR55β. These two isoforms showed 86% homology. Subsequently a third isoform was identified (Pallas *et al*, 1992). Northern blot analysis showed high levels of PR55β in the neuroblastoma cell line LA-N-1 with much lower amounts in the other cell lines examined. These results suggest that PR55β may be part of a brain specific holoenzyme of PP-2A.

Hendrix *et al* (1993) cloned the 72,000 Da subunit of PCS_M from several tissue and cell line cDNA libraries. The human heart cDNA clone encoded a protein with a deduced molecular size of 62,000 Da. while that from brain predicted, and *in vitro* transcription and translation yielded, a protein of 130,000 Da. This protein was identical to the 72,000 Da heart protein from amino acid 45 to the carboxy-terminus with a unique 665 amino acid amino-terminal extension. As the 3' noncoding region of both clones are identical the two proteins are thought to arise from the same gene by the use of different promoters. mRNA for the 72,000 Da protein was found only in heart and muscle while that of the 130,000 Da protein was more widely distributed. The need for two isoforms of the 72,000 Da subunit is unclear although both isoforms contain nuclear localization signals

TABLE 2. *Structure of type-2A protein phosphatases*

Name	Tissue	Ref.	Subunit content			M_r	Proposed structure
H-II	Liver	376	α_2-35	β-65		260	
II	Liver	461	α_2-35	β-69		160	$\alpha_2\beta$
	Shrimp	470	α_2-40	β_2-75		225	$\alpha_2\beta_2$
PCS_{H2}	Skeletal muscle	513	35	65		109	
$2A_2$	Skeletal muscle	484	C-36	A-60		107	
IV	Erythrocyte	490	α-34	β-63		104	αβ
PCS_L	Skeletal muscle	513	35	65		109	
PT-2	Heart	351	38	63		87	
$2A_1$	Skeletal muscle	484	C_2-36	A-60	B-55	202	
IB	Liver	461	α_2-35	β-69	γ-58	260	$\alpha_2\beta\gamma$
PCS_{H1}	Skeletal muscle	513	35	65	55	136	
III	Erythrocyte	490	α-34	β-63	γ-53	177	αβγ
PT-1	Heart	351	38	63	55	137	
$2A_0$	Skeletal muscle	484	C_2-36	A-60	B′-54	181	$\alpha_2\beta\gamma'$
PCS_M	Skeletal muscle	513	35	65	72	162	
I	Erythrocyte	490	α-34	β-63	δ-74	180	αβδ

Table 2: Structure of type 2A protein phosphatases Reproduced from Shenolikar and Nairn (1991)

(Hendrix *et al*, 1993) and PP-2A has been found in rat liver and *Xenopus* oocyte nuclei (Jakes *et al*, 1986; Jessus *et al*, 1989).

Two isoforms of the 65,000 Da subunit have also been identified (Walter *et al*, 1989; Hemmings *et al*, 1990). It is therefore apparent that the possible combinations between the many subunits can give rise to a large number of PP-2A holoenzymes. The variation in subunit composition may influence subcellular distribution and substrate specificity.

1.3.3.1 Regulation of protein phosphatase-2A

The regulation of PP-2A is not well understood. Generally speaking the associated subunits mentioned above seem to inhibit the activity of PP-$2A_c$. For example selective degradation of the 55,000 Da subunit from turkey gizzard by trypsin increased phosphatase activity (Pato and Kerc, 1986). A reduction in activity was noted upon the reassociation of a 60,000 Da subunit of reticulocyte PP-2A with the catalytic subunit (Chen *et al*, 1989). The 65,000 Da subunit was shown to decrease phosphorylase a and histone H2B phosphatase activity but increase histone H1 phosphatase activity (Imaoka *et al*, 1983; Tsuiki *et al*, 1985). The γ subunit of erythrocytes (Table 2) was shown to suppress phosphatase activity towards all substrates examined (Usui *et al*, 1988). PP-$2A_1$ was shown to have a lower activity towards myosin and phosphorylase a than PP-$2A_2$ (Mumby *et al*, 1987; Tamura and Tsuiki, 1980). The activity of PP-$2A_0$ is extremely low, indicating that the B' subunit (54,000 Da) suppresses dephosphorylation more strongly than the B subunit (Ingebritsen *et al*, 1983).

Contrary to most situations, histone H1, phosphorylated by cdc2 kinase, and the microtubule associated protein tau, phosphorylated by p42 MAP kinase, were found to be dephosphorylated more readily by PP-$2A_1$ than by PP-$2A_2$ (Sola *et al*, 1991; Goedert *et al*, 1992). This result was unexpected because the presence of a proline residue C-terminal to a phosphorylated residue prevents dephosphorylation by all serine/threonine protein phosphatase catalytic subunits (Donella-Deana *et*

al, 1990; Agostinis *et al*, 1990). Since this motif represents a part of the consensus sequence for cdc2 and MAP kinase phosphorylation, the associated subunits must make this dephosphorylation possible.

PP-2A is almost completely soluble following centrifugation at 100,000x*g* (Cohen, 1989). However, Sim *et al* (1994) recently showed significant amounts of the enzyme in the particulate fraction of rat forebrain, indicating that the associated subunits may serve to target the phosphatase to specific subcellular regions of the neuron and thus regulate its activity.

Since PP-2A activity can be stimulated *in vitro* by basic proteins and polyamines, Cornwell *et al* (1987) determined the intracellular concentration of the polyamine spermine and found it to be within the range at which it can regulate PP-2A. However, it remains to be determined whether spermine is in fact a physiological regulator of this protein phosphatase.

The phorbol ester TPA was found to stimulate PP-2A activity when applied to mouse skin (Gschwendt *et al*, 1989) whereas PKA inhibited PP-2A activity toward the enzyme acetyl CoA carboxylase by an unknown mechanism (Haystead *et al*, 1990). The cerebellum contains a protein known as G-substrate which is phosphorylated by cGMP-dependent protein kinase (Aswad and Greengard, 1981) and is dephosphorylated by the calcium/calmodulin-dependent protein phosphatase, PP-2B (King *et al*, 1984). When phosphorylated, G-substrate inhibits PP-2A and, to a lesser degree, PP-1. Finally, ceramide, a potential second messenger and the product of sphingomyelin catabolism, activates $PP\text{-}2A_2$ but not $PP\text{-}2A_1$ nor $PP\text{-}2A_c$ (Dobrowsky *et al*, 1993) further indicating the inhibitory nature of the 55,000 Da subunit. These data suggest that, similar to PP-1, PP-2A may be regulated in response to second messengers. In addition to the preceding example, the 55,000 Da subunit was shown to have a regulatory function on PP-2A activity since $PP\text{-}2A_1$ was found to be more active on phosphorylated tau protein than were $PP\text{-}2A_2$ or PCS_M (Drewes *et al*, 1993).

A phosphorylation site which may regulate the association of the 65,000 Da

subunit with PP-2A$_c$ has been identified (Hemmings *et al*, 1990) and the 72,000 Da subunit is a substrate for both casein kinases-1 and -2 (Hendrix *et al*, 1993). One interesting but ill-defined mode of regulation of PP-2A activity is by phosphorylation of the catalytic and 60,000 Da subunits of PP-2A$_2$. Guo and Damuni (1993) showed that a protein kinase in bovine kidney thus phosphorylated and inactivated PP-2A$_2$ by 80%. The mechanism of inhibition was not determined but the protein kinase is itself activated by autophosphorylation, which could be reversed by either PP-2A$_2$ or PP-1$_c$ (Guo *et al*, 1993).

1.3.3.2 Physiological roles of protein phosphatase-2A

Several regulatory enzymes involved in amino acid catabolism, fatty acid synthesis and glycolysis/gluconeogenesis are excellent substrates for PP-2A suggesting a role for this protein phosphatase in these areas of metabolism. The enzymes include pyruvate kinase, 6-phosphofructo-1-kinase, acetyl CoA carboxylase, ATP citrate lyase and phenylalanine hydroxylase (Cohen, 1989).

The β-adrenergic receptor from erythrocytes is dephosphorylated by a protein phosphatase in the subcellular compartment referred to as *light membrane vesicles* which is known to contain PP-2A (Sibley *et al*, 1986). Since the β_2-adrenergic receptor is dephosphorylated *in vitro* by PP-2A and dephosphorylation is thought to regulate receptor recycling this suggests a physiological role for PP-2A in this process.

A physiological role for PP-2A in the regulation of photoreception is implied by the finding that PP-2A is the major rhodopsin phosphatase and that the binding of the protein arrestin to rhodopsin prevented its dephosphorylation by PP-2A (Fowles *et al*, 1989).

An *in vivo* role for PP-2A in protein synthesis was indicated because low concentrations of the protein phosphatase inhibitor okadaic acid (see below) inhibited protein synthesis in reticulocyte lysates (Redpath and Proud, 1989). In addition PP-2A has been shown to dephosphorylate eukaryotic initiation factor eIF-

4E, although the significance of this action remains unknown (Bu and Hagedorn, 1992).

PP-2A$_{\alpha}$ was shown to be an essential component for the *in vitro* transcription of the SV40 large T-antigen (Virshup and Kelly, 1989) and may also function in the regulation of cell growth and division since a related protein phosphatase, called PP-X (see above), is found in all tissues examined and is localized to centrosomes in the nucleus (Brewis *et al*, 1993). PP-2A negatively regulates cdc2 kinase activity (Félix *et al*, 1990) and dephosphorylates histone H1, one of the substrates of this protein kinase (Sola *et al*, 1991).

PP-2A also dephosphorylates B-50, a presynaptic membrane-associated protein whose phosphorylation state is related to release of noradrenaline, the activity of phosphatidylinositol 4-phosphate kinase and long-term potentiation (Han *et al*, 1992).

In addition PP-2A was capable of dephosphorylating abnormally phosphorylated tau protein found in Alzheimer disease brain (Goedert *et al*, 1992; Drewes *et al*, 1993). In a related study, dephosphorylation of MAP2 and tau protein released the inhibition of microtubule polymerization by the phosphorylated isoforms of these proteins (Yamamoto *et al*, 1988). It is unclear, however, if this was a PP-2A or PP-1 mediated event since the PP-2A preparation contained low (10%) amounts of PP-1. This same group also reported that the PP-2A from rat brain which dephosphorylates tau protein is inhibited by aluminum (Yamamoto *et al*, 1990) thus raising the possibility that the abnormal phosphorylation of certain cytoskeletal proteins after aluminum administration (Johnson and Jope, 1988) may be due to the inhibition of protein phosphatase activity.

PP-2A purified from rat liver nuclei dephosphorylated the *cAMP regulatory element binding protein* (Wadzinski *et al*, 1993). This finding is significant since, in most other *in vitro* studies, cytoplasmic PP-2A is used.

Finally, PP-2A, as well as PP-1, were recently shown to regulate NMDA

currents in cultured hippocampal cells (Wang *et al*, 1994). NMDA channels allow calcium ions, the divalent cation required by PP-2B (see below), into the cell. Both DARPP-32, a PP-1 inhibitor, and G-substrate, a PP-2A inhibitor, are excellent substrates for PP-2B (see below). These data suggest a complex interplay between several protein phosphatases in the regulation of NMDA currents.

1.3.4 Protein phosphatase-2B: Structure, regulation and physiological roles

All forms of PP-2B are heterodimers consisting of a 1:1 complex between a 61,000 Da (A) polypeptide and a 15,000-16,000 Da (B) polypeptide. The A subunit possesses the catalytic activity (Winkler *et al*, 1984; Tallant and Cheung, 1984) and contains the calmodulin binding site (Sharma *et al*, 1979) while the B subunit contains the calcium binding site (Aitken *et al*, 1984). Two forms of the A subunit from human brain have been cloned which are the products of alternate splicing of a common transcript (Guerini and Klee, 1989). A third isoform of the A subunit cloned from *Drosophila* encodes a protein, termed PP-$2B_W$, with 54% homology to the brain isoforms (da Cruz e Silva and Cohen, 1989). The B subunits from bovine (Aitken *et al*, 1984) and human (Guerini *et al*, 1989) brain have been cloned and differ in only three amino acids. Interestingly, the B subunit was shown to be myristoylated on its amino-terminal glycine residue (Aitken *et al*, 1982) which may explain its localization in both the particulate and soluble fractions in brain whereas in other tissues the enzyme is almost completely soluble (Klee and Cohen, 1988). In some cases the particulate form of PP-2B cannot be extracted with non-ionic detergents leading to the suggestion that the enzyme may be associated with the cytoskeleton (Alexander *et al*, 1988).

PP-2B is highly concentrated in brain with levels approaching 1% of total brain protein. In other tissues it is present at just 0.03% of total protein. PP-2B activities in brain and skeletal muscle extracts are similar (Ingebritsen *et al*, 1983) indicating that the brain form of the enzyme has a lower specific activity than that of skeletal muscle. Due to its high concentration in brain, this species of PP-2B is

the best studied and is referred to as calcineurin.

A model for the synergistic activation of calcineurin by calcium and calmodulin has been developed (see Ballou and Fischer, 1986, Klee and Cohen, 1988, for details). Briefly, the AB dimer is inactive in the absence of calcium. Binding of calcium to the B subunit partially activates the enzyme which is further activated by the binding of calmodulin. Complete activation of the enzyme occurs in the presence of nickel or manganese ions (Stewart *et al*, 1983) which are thought to exert their effects by inducing a slow conformational change in the A subunit (Pallen and Wang, 1984).

PP-2B in brain is thought to dephosphorylate DARPP-32, I1 and G-substrate (King *et al*,1984), hence influencing the activities of PP-1 and PP-2A (see above). Depolarization of synaptosomes leads to the rapid dephosphorylation of several proteins including dephosphin which is thought to be the trigger in neurotransmitter release (Robinson, 1992). The phosphorylation state of dephosphin is thought to be controlled by PP-2B (Sim *et al*, 1991). Calcineurin has been shown to dephosphorylate abnormally phosphorylated tau protein in Alzheimer's disease brain (Drewes *et al*, 1993). *In vitro*, calcineurin was capable of dephosphorylating the muscarinic acetylcholine receptor from rat brain restoring ligand binding (Rosenbaum *et al*, 1987). PP-2B has also been implicated in the regulation of sodium (Rossie and Catterall, 1989) and calcium (Armstrong, 1989) channels. Finally, PP-2B was shown to dephosphorylate the synaptic membrane protein B-50 (Liu and Storm, 1989).

1.3.5 Protein phosphatase-2C: Structure, regulation and physiological roles

PP-2C is a monomeric enzyme of 43,000 Da which requires magnesium or manganese for activity (Cohen, 1989; Shenolikar and Nairn, 1991). Two isoforms of the enzyme, PP-2Cα and PP-2Cβ, have been cloned from rabbit muscle and liver and show a limited degree of homology (McGowan and Cohen, 1987; Wenk *et al*, 1992).

Besides the requirement for magnesium or manganese, other factors that may be involved in the regulation of the activity of this protein phosphatase are not known. Recently PP-2Cα was shown to be a phosphoprotein whose phosphorylation *in vivo* by casein kinase-2 may be physiologically relevant (Kobayashi *et al*, 1993). Since PP-2Cβ does not contain a casein kinase-2 consensus sequence (Wenk *et al*, 1992) perhaps the two isoforms of this protein phosphatase are regulated by separate mechanisms.

The physiological roles of PP-2C remain to be elucidated. Higher levels of the protein phosphatase are found in brain and liver than in skeletal muscle (Cohen, 1989). In this respect it is noteworthy that PP-2C was capable of dephosphorylating tau protein phosphorylated by PKA but not abnormally phosphorylated tau found in Alzheimer's disease brain (Gong *et al*, 1994) implying that this phosphatase may be involved in the regulation of the phosphorylation state of tau under non-pathological conditions.

1.3.6 Non-protein inhibitors of protein phosphatases

Several cytotoxic compounds have recently been identified which inhibit PP-1, PP-2A and PP-2B. Okadaic acid (OA), a tumour promoter found in the marine sponge *Halichondria okadai*, is a polyether fatty acid which inhibits PP-2A and PP-1 with IC_{50} values of 0.1-1 nM and 10-500 nM, respectively (Bialojan and Takai, 1988; Ishihara *et al*, 1989). OA was also shown to inhibit PP-2B at high concentrations (IC_{50} 4-5 μM; Bialojan and Takai, 1988). Since OA does not inhibit a protein phosphatase encoded by the bacteriophage lambda genome which is homologous to both $PP\text{-}1_c$ and $PP\text{-}2A_c$ but lacks the carboxy-terminal 100 amino acids (Cohen and Cohen, 1989), OA is thought to inhibit these protein phosphatases by binding to the carboxy-terminal third of the catalytic subunits (Bollen and Stalmans, 1992).

Another marine sponge compound, calyculin A (clA), was found to be a potent inhibitor of both PP-1 and PP-2A with a similar IC_{50} of 0.5-1 nM (Ishihara

et al, 1989). Interestingly, clA contains a phosphate monoester (Kateo *et al*, 1986) which may be involved in the mechanism of inhibition.

Tautomycin, a compound structurally similar to OA, is found in the bacterium *Streptomyces verticullatus* and inhibits PP-1 more potently than PP-2A (IC_{50}s of 0.16 nM and 0.4 nM, respectively; MacKintosh and Klumpp, 1990). PP-2B was inhibited by this compound only at extremely high concentrations (IC_{50}>30 μM).

Phenothiazines and derivatives such as trifluoperazine at 100 μM inhibit PP-2B specifically (Stewart *et al*, 1983). Aside from the general protein phosphatase inhibitor orthovanadate, no specific inhibitors of PP-2C are presently known. Use of combinations of the inhibitors mentioned above, therefore, can help determine (or eliminate) the protein phosphatase(s) regulating a particular function.

1.3.7 Protein phosphatases and neurofilaments

A limited number of studies have been carried out which examine the actions and the effects of protein phosphatases on NFs. Hisanaga and Hirokawa (1989) showed that treatment of bovine NFs with alkaline phosphatase had no effect on the number of projections on the filaments nor on the ability of the filaments to form crossbridges. Minami and Sakai (1985) showed that dephosphorylation of NFs, and in particular NF-H, suppressed the activity of NFs in promoting tubulin polymerization. Neither PP-1, PP-2A, PP-2B nor PP-2C were capable of inducing the dephosphorylation-dependent interaction between NFs and microtubules (Hisanaga *et al*, 1993).

Activation of kainic acid receptors *in vivo* caused a decrease in NF-H phosphorylation (Wang *et al*, 1992,1994). Since this treatment also causes an influx of calcium ions it would be of interest to determine whether PP-2B is involved in this effect.

Identification of protein phosphatases in neural tissue has led to more questions than answers. Rats which were chronically fed ethanol showed an

increase in NF phosphorylation as well as a decrease in protein phosphatase activity in spinal cord extracts (Guru *et al*, 1991). Since ethanol was not inhibitory to spinal cord protein phosphatase activity *in vitro* (Shetty *et al*, 1992) it was suggested that protein kinase activity was raised and phosphorylation of modulators of protein phosphatases caused inhibition of phosphatase activity (see above). The phosphatase activity in rat spinal cords was shown to be inhibited by aluminum and, interestingly, trifluoperazine, which may indicate a PP-2B-like activity (Shetty *et al*, 1992). However, since PP-2B is not inhibited by aluminum, several protein phosphatases may be found in spinal cord. In this respect it is noteworthy that NF phosphorylation is increased following aluminum intoxication (Bizzi and Gambetti, 1986) and in perikaryal regions in Alzheimer's disease brain (Sternberger *et al*, 1985), a disease in which Gong *et al* (1993) showed a decrease in PP-1 and PP-2A activity. Since Honkanen *et al* (1991) identified a protein phosphatase, distinct from the aforementioned classes, which they called PP-3 it would be of interest to determine the activity of this enzyme in the presence of aluminum and trifluoperazine. It is, therefore, clear that more work is needed to examine the involvement of protein phosphatases on NF phosphorylation under normal and pathological conditions.

References

Abeele,C.V., Vandenheede,J.R. and Merlevede,W. (1987) *J. Biol. Chem.* **262**, 14,086-14,089

Agostinis,P., Vandenheede,J.R., Goris,J., Meggio,F., Pinna,L.A. and Merlevede,W. (1987) *FEBS Lett.* **224**, 385-390

Agostinis,P., Goris,J., Pinna,L.A., Marchiori,F., Perich,J.W., Meyer,H.E. and Merlevede,W. (1990) *Eur. J. Biochem.* **189**, 235-241

Aitken,A., Bilham,T. and Cohen,P. (1982) *Eur. J. Biochem.* **126**, 235-246

Aitken,A., Cohen,P., Santikarn,S., Williams,D.H., Calder,A.G. and Klee,C. (1982) *FEBS Lett.* **150**, 314-318

Aitken,A. and Cohen,P. (1982) *FEBS Lett.* **147**, 54-58

Aitken,A., Klee,C. and Cohen,P. (1984) *Eur. J. Biochem.* **139**, 663-671

Albers,K. and Fuchs,E. (1987) *J. Cell Biol.* **105**, 791-806

Alemany,S. and Cohen,P. (1986) *FEBS Lett.* **198**, 194-202

Alessi,D.R., Street,A.J., Cohen,P. and Cohen,P.T.W. (1993) *Eur. J. Biochem.* **213**, 1055-1066

Alexander,D.R., Hexham,J.M. and Crumpton,M.J. (1988) *Biochem. J.* **256**, 885-892

Allan,V.J., Vale,R.D. and Navone,F. (1991) in *The Neuronal Cytoskeleton* pp.257-

282 ed. R.D. Burgoyne, Wiley-Liss,N.Y.

Ando,S., Tanabe,K., Gonda,Y., Sato,C. and Inagaki,M. (1989) *Biochemistry* **28**, 2974-2979

Angelides,K.J., Smith,K.E. and Takeda,M. (1989) *J. Cell Biol.* **108**, 1495-1506

Angelov,I. (1994) *Arch. Biochem. Biophys.* **310**, 97-107

Arino,J., Woon,C.W., Brautigan,D.L., Miller,T.B. and Johnson,G.L (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4252-4256

Armstrong,D.L. (1989) *Trends Neurosci.* **12**, 117-122

Aswad,D.W. and Greengard,P. (1981) *J. Biol. Chem.* **256**, 3487-3493

Bader,B.L., Magin,T.M., Hatzfeld,M. and Franke,W.W. (1986) *EMBO J.* **5**, 1865-1875

Balin,B.J. and Lee,V.M.-Y. (1991) *Brain Res.* **556**, 196-208

Balin,B.J., Clark,E.A., Trojanowski,J.Q. and Lee,V.M.-Y. (1991) *Brain Res.* **556**, 181-195

Ballou,L.M., Brautigan,D.L. and Fischer,E.H. (1983) *Biochemistry* **22**, 3393-3399

Ballou,L.M., Jeno,P. and Thomas,G. (1988) *J. Biol. Chem.* **263**, 1188-1194

Bamburg,J.R. and Bernstein,B.W. (1991) in *The Neuronal Cytoskeleton* pp.121-160,

ed. R.D. Burgoyne, Wiley-Liss, N.Y.

Bamburg,J.R., Harris,H.E. and Weeds,A.G. (1980) *FEBS Lett.* **121**, 178-182

Banerjee,A. and Ludeña,R.F. (1987) *FEBS Lett.* **219**, 103-107

Barker,H.M., Jones,T.A., da Cruz e Silva,E.F., Spurr,N.K., Sheer,D. and Cohen,P.T.W. (1990) *Genomics* **7**, 159-166

Barker,H.M., Craig,S.P., Spurr,N.K. and Cohen,P.T.W. (1993) *Biochim. Biophys. Acta* **1178**, 228-233

Barker,H.M., Brewis,N.D., Street,A.J., Spurr,N.K. and Cohen,P.T.W. (1994) *Biochim. Biophys. Acta* **1220**, 212-218

Barra,H.S., Arce,C.E., Rodriguez,J.A. and Caputto,R. (1973) *J. Neurochem.* **20**, 97-108

Beaudette,K.N., Lew,J. and Wang,J.H. (1993) *J. Biol. Chem.* **268**, 20,825-20,830

Berndt,N., Campbell,D.G., Caudwell,F.B., Cohen,P. da Cruz e Silva,E.F., da Cruz e Silva,O.B. and Cohen,P.T. (1987) *FEBS Lett.* **223**, 340-346

Bernhardt,R. and Matus,A. (1984) *J. Comp. Neurol.* **226**, 203-221

Beullens,M., Van Eynde,A., Stalmans,W. and Bollen,M. (1992) *J. Biol. Chem.* **267**, 16,538-16,544

Beullens,M., Van Eynde,A., Bollen,M. and Stalmans,W. (1993) *J. Biol. Chem.* **268**,

13,172-13,177

Bialojan,C. and Takai,A. (1988) *Biochem. J.* **256**, 283-290

Binder,L.I., Frankfurter,A. and Rebhun,L.I. (1985) *J. Cell Biol.* **101**, 1371-1378

Bizzi,A. and Gambetti,P. (1986) *Acta Neuropathol.* **71**, 154-158

Bollen,M., Vandenheede,J.R., Goris,J. and Stalmans,W. (1988) *Biochim. Biophys. Acta* **969**, 66-77

Bollen,M. and Stalmans,W. (1992) *Critic. Rev. Biochem. Mol. Biol.* **27**, 227-281

Bollen,M., Depaoli-Roach,A.A. and Stalmans,W. (1994) *FEBS Lett.* **344**, 196-200

Bonifas,J.M., Rothman,A.L. and Epstein,E.H. (1991) *Science* **254**, 1202-1205

Brautigan,D.L., Shriner,C.L. and Gruppuso,P.A. (1985) *J. Biol. Chem.* **260**, 4295-4302

Brautigan,D.L., Gruppuso,P.A. and Mumby,M. (1986) *J. Biol. Chem.* **261**, 14,924-14,928

Brewis,N.D., Street,A.J., Prescott,A.R. and Cohen,P.T.W. (1993) *EMBO J.* **12**, 987-996

Bu,X. and Hagedorn,C.H. (1992) *FEBS Lett.* **301**, 15-18

Burgoyne,R.D. (1991) in *The Neuronal Cytoskeleton* pp.75-91 ed. R.D. Burgoyne,

Wiley-Liss,N.Y.

Caputo,C.B., Sygowski,L.A., Brunner,W.F., Scott,C.W. and Salama,A.I. (1989) *Biochim. Biophys. Acta* **1012**, 299-307

Carden,M.J., Schlaepfer,W.W. and Lee,V.M.-Y. (1985) *J. Biol. Chem.* **260**, 9805-9817

Carden,M.J., Trojanowski,J.Q., Schlaepfer,W.W. and Lee,V.M.-Y. (1987) *J. Neurosci.* **7**, 3489-3504

Carlier,M. and Pantaloni,D. (1981) *Biochemistry* **20**, 1918-1924

Caudwell,F.B., Hiraga,A. and Cohen,P. (1986) *FEBS Lett.* **194**, 85-90

Chang,L.Y. and Huang,L.C. (1980) *Acta Neuroendocrinol.* **95**, 427-436

Charbonneau,H. and Tonks,N.K. (1992) *Annu. Rev. Cell Biol.* **8**, 463-493

Chen,J., Kanai,Y., Cowan,N.J. and Hirokawa,N. (1992) *Nature* **360**, 674-677

Chen,S.C., Kramer,G. and Hardesty,B. (1989) *J. Biol. Chem.* **264**, 7267-7275

Chessa,G., Borin,G., Marchiori,F., Meggio,F., Brunati,A.M. and Pinna,L.A. (1983) *Eur. J. Biochem.* **135**, 609-614

Chin,S.S.M. and Liem,R.K.H. (1989) *Eur. J. Biochem.* **50**, 475-490

Chin,S.S.M. and Liem,R.K.H. (1990) *J. Neurosci.* **10**, 3714-3726

Chin,S.S.M., Macioce,P. and Liem,R.K.H. (1991) *J. Cell Sci.* **99**, 335-350

Chin,T.K., Harding,S.E. and Eagles,P.A.M. (1989) *Biochem. J.* **264**, 53-60

Ching,G.Y. and Liem,R.K.H. (1993) *J. Cell Biol.* **122**, 1323-1335

Chisholm,A.A. and Cohen,P. (1988) *Biochim. Biophys. Acta* **968**, 392-400

Choo,Q.L. and Bray,D. (1978) *J. Neurochem.* **31**, 217-224

Chou,Y.-H., Rosevear,E. and Goldman,R.D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1885-1889

Chou,Y.-H., Bischof,J.R., Beach,D. and Goldman,R.D. (1990) *Cell* **62**, 1063-1071

Chou,Y.-H., Ngai,K.-L. and Goldman,R.D. (1991) *J. Biol. Chem.* **266**, 7325-7328

Chun,Y.-S., Shima,H., Nagasaki,K., Sugimura,T. and Nagao,M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3319-3323

Ciment,G., Resler,A. Letourneau,P.C. and Weston,J.A. (1986) *J. Cell Biol.* **102**, 246-251

Cleveland,D.W., Spiegelman,B. and Kirschner,M. (1979) *J. Biol. Chem.* **254**, 12,670-12,678

Cleveland,D.W., Lopata,M.A., MacDonald,R.J., Cowan,N.J., Rutter,W.J. and Kirschner,M.W. (1980) *Cell* **20**, 95-105

Cohen,P. (1989) *Annu. Rev. Biochem.* **58**, 453-508

Cohen,P.T. and Cohen,P. (1989) *Biochem. J.* **260**, 931-934

Cohlberg,J.A., Hajarian,H. and Sainte-Marie,S. (1987) *J. Biol. Chem.* **262**, 17,009-17,015

Cornwell,T., Mehta,P. and Shenolikar,S. (1987) *J. Cyc. Nuc. Prot. Phosph. Res.* **12**, 373-383

Coté,F., Collard,J.-F. and Julien,J.-P. (1993) *Cell* **73**, 35-46

Coulombe,P.A. and Fuchs,E. (1990) *J. Cell Biol.* **111**, 153-169

Coulombe,P.A., Hutton,M.E., Letai,A., Hebert,A., Paller,A.S. and Fuchs,E. (1991) *Cell* **66**, 1301-1311

da Cruz e Silva,O.B., Alemany,S., Campbell,D.G. and Cohen,P.T. (1987) *FEBS Lett.* **221**, 415-422

da Cruz e Silva,O.B. and Cohen,P.T. (1987) *FEBS Lett.* **226**, 176-178

da Cruz e Silva,O.B., da Cruz e Silva,E.F. and Cohen,P.T.W. (1988) *FEBS Lett.* **242**, 106-110

da Cruz e Silva,O.B. and Cohen,P.T.W. (1989) *Biochim. Biophys. Acta* **1009**, 293-296

De Camilli,P., Miller,P.E., Navone,F., Theurkauf,W.E. and Vallee,R.B. (1984)

Neuroscience **11**, 817-846

Dent,P., Campbell,D.G., Hubbard,M.J. and Cohen,P. (1989) *FEBS Lett.* **248**, 67-72

Dent,P., Campbell,D.G., Caudwell,F.B. and Cohen,P. (1990) *FEBS Lett.* **259**, 281-286

Dent,P., Lavoinne,A., Nakielny,S. Caudwell,F.B. and Cohen,P. (1990) *Nature* **348**, 302-308

DePaoli-Roach,A.A. (1984) *J. Biol. Chem.* **259**, 12,144-12,152

Dessev,G. and Goldman,R. (1988) *Dev. Biol.* **130**, 543-550

Dobrowsky,R.T., Kamibayashi,C., Mumby,M.C. and Hannun,Y.A. (1993) *J. Biol. Chem.* **268**, 15,523-15,530

Dombrádi,V., Axton,J.M., Barker,H.M. and Cohen,P.T.W. (1990) *FEBS Lett.* **275**, 39-43

Donella-Deana,A., McGowan,C.H., Cohen,P., Marchiori,F., Meyer,H.E. and Pinna,L.A. (1990) *Biochim. Biophys. Acta* **1051**, 199-202

Doonan,J.H. and Morris,N.R. (1989) *Cell* **57**, 987-996

Dosemeci,A., Floyd,C.C. and Pant,H.C. (1990) *Cell. Mol. Neurobiol.* **10**, 369-382

Dosemeci,A. and Pant,H.C. (1992) *Biochem. J.* **282**, 477-481

Drewes,G., Mandelkow,E.-M., Baumann,K., Goris,J., Merlevede,W. and Mandelkow,E. (1993) *FEBS Lett.* **336**, 425-432

Dunkley,P.R. and Robinson,P.J. (1983) *Neurochem. Res.* **8**, 865-871

Eckert,B.S., Daley,R.A. and Parysek,L.M. (1982) *J. Cell Biol.* **92**, 575-578

Elbrecht,A., DiRenzo,J., Smith,R.G. and Shenolikar,S. (1990) *J. Biol. Chem.* **265**, 13,415-13,418

Eriksson,J.E., Opal,P. and Goldman,R.D. (1992) *Curr. Opin. Cell Biol.* **4**, 99-104

Eriksson,J.E., Brautigan,D.L., Vallee,R., Olmsted,J., Fujiki,H. and Goldman,R.D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11,093-11097

Ernst,V., Levin,D.H., Foulkes,J.G. and London,I.M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7092-7096

Eyer,J. and Leterrier,J.-F. (1988) *Biochem. J.* **252**, 655-660

Eyer,J. and Peterson,A. (1994) *Neuron* **12**, 389-405

Félix,M.-A., Cohen,P. and Karsenti,E. (1990) *EMBO J.* **9**, 675-683

Fernandez,A., Brautigan,D.L., Mumby,M. and Lamb,N.J.C. (1990) *J. Cell Biol.* **111**, 103-112

Fernandez,A., Brautigan,D.L. and Lamb,N.J.C. (1992) *J. Cell Biol.* **116**, 1421-1430

Field,D.J., Collins,R.A. and Lee,J.C. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4041-4045

Fiol,C.J., Haseman,J.H., Wang,H., Roach,P.J., Roeske,R.W., Kowalczuk,M. and DePaoli-Roach,A.A. (1988) *Arch. Biochem. Biophys.* **267**, 797-802

Flanagan,M.D. and Lin,S. (1979) *J. Neurochem.* **32**, 1037-1046

Fliegner,K.H. and Liem,R.K.H. (1991) *Int. Rev. Cytol.* **131**, 109-167

Floyd,C.C., Grant,P., Gallant,P.E. and Pant,H.C. (1991) *J. Biol. Chem.* **266**, 4987-4994

Foulkes,J.G., Strada,S.J., Henderson,P.J.F. and Cohen,P. (1983) *Eur. J. Biochem.* **132**, 309-313

Fowles,C., Akhtar,M. and Cohen,P. (1989) *Biochemistry* **28**, 9385-9391

Frappier,T., Regnouf,F. and Pradel,L.A. (1987) *Eur. J. Biochem.* **169**, 651-657

Friedman,D.L. (1986) *Biochem. Biophys. Res. Comm.* **134**, 1372-1378

Gard,D.L. and Kirschner,M.W. (1985) *J. Cell Biol.* **100**, 764-774

Gardner,E.E., Dahl,D. and Bignami,A. (1984) *J. Neurosci. Res.* **11**, 145-155

Geisler,N. and Weber,K. (1981) *J. Mol. Biol.* **151**, 565-571

Geisler,N. and Weber,K. (1988) *EMBO J.* **7**, 15-20

Geisler,N., Plessmann,U. and Weber,K. (1985) *FEBS Lett.* **182**, 475-478

Geisler,N., Kaufmann,E. and Weber,K. (1985) *J. Mol. Biol.* **182**, 173-177

Geisler,N., Vandekerckhove,J. and Weber,K. (1987) *FEBS Lett.* **221**, 403-407

Geisler,N., Hatzfeld,M. and Weber,K. (1989) *Eur. J. Biochem.* **183**, 441-447

Gelfand,V.I. and Bershadsky,A.D. (1991) *Annu. Rev. Cell Biol.* **7**, 93-116

Georges,E., Lefebvre,S. and Mushynski,W.E. (1986) *J. Neurochem.* **47**, 477-483

Georges,E., Trifaró,J.M. and Mushynski,W.E. (1987) *Neurosci.* **22**, 753-763

Georges,E. and Mushynski,W.E. (1987) *Eur. J. Biochem.* **165**, 281-287

Georges,E., Lindenbaum,M.H., Sacher,M.G., Tifaró,J.-M. and Mushynski,W.E. (1989) *J. Neurochem.* **52**, 1156-1161

Georgieff,I.S., Liem,R.K.H., Mellado,W., Nunez,J. and Shelanski,M.L. (1991) *J. Cell Sci.* **100**, 55-60

Gill,S.R., Wong,P.C., Monteiro,M.J. and Cleveland,D.W. (1990) *J. Cell Biol.* **111**, 2005-2019

Girault,J.A., Hemmings,H.C.Jr., Williams,K.R., Nairn,A.C. and Greengard,P. (1989) *J. Biol. Chem.* **264**, 21,748-21,759

Giuliano,K.A., Khatib,F.A., Hayden,S.M., Daoud,E.W.R., Adams,M.E.,

Amorese,D.A., Bernstein,B.W. and Bamburg,J.R. (1988) *Biochemistry* **27**, 8931-8938

Goedert,M., Cohen,E.S. and Cohen,P. (1992) *FEBS Lett.* **312**, 95-99

Goldman,R.D., Goldman,A.E., Green,K.J., Jones,J.C.R., Jones,S.M. and Yang,H.-Y. (1986) *J. Cell Sci. Suppl.* **5**, 69-97

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

Gonda,Y., Nishizawa,K., Ando,S., Kitamura,S., Minoura,Y., Nishi,Y. and Inagaki,M. (1990) *Biochem. Biophys. Res. Comm.* **167**, 1316-1325

Gong,C.-X., Singh,T.J., Grundke-Iqbal,I. and Iqbal,K. (1993) *J. Neurochem.* **61**, 921-927

Gong,C.-X., Grundke-Iqbal,I., Damuni,Z. and Iqbal,K. (1994) *FEBS Lett.* **341**, 94-98

Goris, J., Defryn,G. and Merlevede,W. (1979) *FEBS Lett.* **99**, 279-282

Gotow,T., Takeda,M., Tanaka,T. and Hashimoto,P.H. (1992) *Eur. J. Cell Biol.* **58**, 331-345

Gotow,T. and Tanaka,J. (1994) *J. Neurosci. Res.* **37**, 691-713

Grafstein,B. and Forman,D.S. (1980) *Physiol. Rev.* **60**, 1167-1283

Grant,N.J. and Aunis,D. (1990) *Eur. J. Cell Biol.* **52**, 36-46

Gratecos,D., Detwiler,T.C., Hurd,S. and Fischer,E.H. (1977) *Biochemistry* **16**, 4812-4817

Green,D.D., Yang,S.I. and Mumby,M.C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4880-4884

Gschwendt,M., Kittstein,W., Mieskes,G. and Marks,F. (1989) *FEBS Lett.* **257**, 357-360

Guan,R.J., Khatra,B.S. and Cohlberg,J.A. (1991) *J. Biol. Chem.* **266**, 8262-8267

Guan,R.J., Hall,F.L. and Cohlberg,J.A. (1992) *J. Neurochem.* **58**, 1365-1371

Guerini,D. and Klee,C. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9183-9187

Guerini,D., Krinks,M.H., Sikela,J.M., Hahn,W.E. and Klee,C. (1989) *DNA* **8**, 675-682

Gundersen,G.G. and Bulinski,J.C. (1986) *Eur. J. Cell Biol.* **42**, 288-294

Guo,H. and Damuni,Z. (1993) *Proc. Natl. Acad. Sci USA* **90**, 2500-2504

Guo,H., Reddy,S.A.G. and Damuni,Z. (1993) *J. Biol. Chem.* **268**, 11,193-11,198

Guru,S.C., Shetty,K.T. and Shankar,S.K. (1991) *Neurochem. Res.* **16**, 1193-1197

Hall,J.L., Dudley,L., Dobner,P.R., Lewis,S.A. and Cowan,N.J. (1983) *Mol. Cell Biol.* **3**, 854-862

Halpain,S., Girault,J.A. and Greengard,P. (1990) *Nature* **343**, 369-372

Han,Y., Wang,W., Schlender,K.K., Ganjeizadeh,M. and Dokas,L.A. (1992) *J. Neurochem.* **59**, 364-374

Harada,A., Oguchi,K., Okabe,S., Kuno,J., Terada,S., Ohshima,T., Sato-Yoshitake,R., Takei,Y., Noda,T. and Hirokawa,N. (1994) *Nature* **369**, 488-491

Harris,J., Ayyub,C. and Shaw,G. (1991) *J. Neurosci.* **30**, 47-62

Hatano,S. and Oosawa,F. (1966) *Biochim. Biophys. Acta* **127**, 488-498

Hatzfeld,M. and Weber,K. (1991) *J. Cell Sci.* **99**, 351-362

Haystead,T.A.J., Moore,F., Cohen,P. and Hardie,D.G. (1990) *Eur. J. Biochem.* **187**, 199-205

Heald,R. and McKeon,F. (1990) *Cell* **61**, 579-589

Hecht,N.B., Distel,R.J., Yelick,P.C., Tanhauser,S.M., Driscoll,C.E., Goldberg,E. and Tung,K.S.K. (1988) *Mol. Cell Biol.* **8**, 996-1000

Heimann,R., Shelanski,M.L. and Liem,R.K.H. (1985) *J. Biol. Chem.* **260**, 12,160-12,166

Heins,S., Wong,P.C., Müller,S., Goldie,K., Cleveland,D.W. and Aebi,U. (1993) *J. Cell Biol.* **123**, 1517-1533

Hellmich,M.R., Pant,H.C., Wada,E. and Battey,J.F. (1992) *Proc. Natl. Acad. Sci.*

USA **89**, 10,867-10,871

Hemmings,B.A., Yellowlees,D., Kernohan,J.C. and Cohen,P. (1981) *Eur. J. Biochem.* **119**, 443-451

Hemmings,B.A., Resink,T.J. and Cohen,P. (1982) *FEBS Lett.* **150**, 319-324

Hemmings,H.C.Jr., Nairn,A.C., Aswad,D.W. and Greengard,P. (1984a) *J. Neurosci* **4**, 99-110

Hemmings,H.C.Jr., Nairn,A.C. and Greengard,P. (1984b) *J. Biol. Chem.* **259**, 14,491-14,497

Hemmings,H.C.Jr., Greengard,P., Tung,H.Y. and Cohen,P. (1984c) *Nature* **310**, 503-505

Hemmings,H.C.Jr. and Greengard,P. (1986) *J. Neurosci.* **6**, 1469-1481

Hemmings,B.A., Adams-Pearson,C., Maurer,F., Müller,P., Goris,J., Merlevede,W., Hofsteenge,J. and Stone,S.R. (1990) *Biochemistry* **29**, 3166-3173

Hemmings,H.C.Jr., Nairn,A.C., Elliot,J. and Greengard,P. (1990) *J. Biol. Chem.* **265**, 20,369-20,376

Hendrix,P., Mayer-Jaekel,R.E., Cron,P., Goris,J., Hofsteenge,J., Merlevede,W. and Hemmings,B.A. (1993) *J. Biol. Chem.* **268**, 15,267-15,276

Hendrix,P., Turowski,P., Mayer-Jaekel,R.E., Goris,J., Hofsteenge,J., Merlevede,W. and Hemmings,B.A. (1993) *J. Biol. Chem.* **268**, 7330-7337

Hermann,J., Cayla,X., Dumortier,K., Goris,J., Ozon,R. and Merlevede,W. (1988) *Eur. J. Biochem.* **173**, 17-25

Hill, T. and Carlier,M.F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7234-7238

Hiraga,A. and Cohen,P. (1986) *Eur. J. Biochem.* **161**, 763-769

Hirokawa,N., Hisanaga,S.-I. and Shiomura,Y. (1988) *J. Neurosci.* **8**, 2769-2779

Hisanaga,S. and Hirokawa,N. (1988) *J. Mol. Biol.* **202**, 297-305

Hisanaga,S. and Hirokawa,N. (1989) *J. Neurochem.* **9**, 959-966

Hisanaga,S. and Hirokawa,N. (1990) *J. Biol. Chem.* **265**, 21,852-21,858

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

Hisanaga,S., Gonda,Y., Inagaki,M., Ikai,A. and Hirokawa,N. (1990) *Cell Regul.* **1**, 237-248

Hisanaga,S., Kusubata,M., Okumura,E. and Kishimoto,T. (1991) *J. Biol. Chem.* **266**, 21,798-21,803

Hisanaga,S., Yasugawa,S., Yamakawa,T., Miyamoto,E., Ikebe,M., Uchiyama,M. and Kishimoto,T. (1993) *J. Biochem.* **113**, 705-709

Hisanaga,S., Matsuoka,Y., Nishizawa,K., Saito,T., Inagaki,M. and Hirokawa,N. (1994) *Mol. Biol. Cell* **5**, 161-172

Hoar,P.E., Pato,M.D. and Kerrick,W.G.L. (1985) *J. Biol. Chem.* **260**, 8760-8764

Hoffman,P.N. and Lasek,R.J. (1975) *J. Cell Biol.* **66**, 351-356

Hoffman,P.N., Griffin,J.W. and Price,D.L. (1984) *J. Cell Biol.* **99**, 705-714

Hoffman,P.N., Cleveland,D.W., Griffin,J.W., Landers,P.W., Cowan,N.J. and Price,D.L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3472-3476

Hollander,B.A. and Bennett,G.S. (1992) *Brain Res.* **599**, 237-245

Hollander,B.A., Ayyub,C., Shaw,G. and Bennett,G.S. (1993) *J. Neurochem.* **51**, 2115-2123

Holly,S., Laczkó,I., Fasman,G.D. and Hollósi,M. (1993) *Biochem. Biophys. Res. Comm.* **197**, 755-762

Holmes,C.F., Kuret,J., Chisholm,A.A. and Cohen,P. (1986) *Biochim. Biophys. Acta* **870**, 408-416

Honkanen,R.E., Zwiller,J., Daily,S.L., Khatra,B.S., Dukelow,M. and Boynton,A.L. (1991) *J. Biol. Chem.* **266**, 6614-6619

Huang,F.L. and Glinsmann,W.H. (1976) *Eur. J. Biochem.* **70**, 419-426

Huang,F.L., Tao,S. and Glinsmann,W.H. (1977) *Biochem. Biophys. Res. Comm.* **78**, 615-623

Hubbard,M.J. and Cohen,P. (1989) *Eur. J. Biochem.* **180**, 457-465

Hubbard,M.J., Dent,P., Smythe,C. and Cohen,P. (1990) *Eur. J. Biochem.* **189**, 243-249

Huber,G. and Matus,A. (1984) *J. Cell Biol.* **98**, 777-781

Huber,G., Alaimo-Beuret,D. and Matus,A. (1985) *J. Cell Biol.* **100**, 496-507

Huxley,H.E. (1963) *J. Mol. Biol.* **7**, 281-308

Imaoka,T., Imazu,M., Usui,H., Kinohara,N. and Takeda,M. (1983) *J. Biol. Chem.* **258**, 1526-1535

Imazu,M., Imaoka,T., Usui,H. and Takeda,M. (1979) *Biochem. Biophys. Res. Comm.* **84**, 777-785

Imazu,M., Imaoka,T., Usui,H, Kinohara,N. and Takeda,M. (1981) *J. Biochem.* **90**, 851-862

Inagaki,M., Nishi,Y., Nishizawa,K., Matsuyama,M. and Sato,C. (1987) *Nature* **328**, 649-652

Inagaki,M., Gonda,Y., Matsuyama,M., Nishizawa,K., Nishi,Y. and Sato,C. (1988) *J. Biol. Chem.* **263**, 5870-5978

Inagaki,M., Gonda,Y., Ando,S., Kitamura,S., Nishi,Y. and Sato,C. (1989) *Cell Struct. Funct.* **14**, 279-286

Inagaki,M., Gonda,Y., Nishizawa,K., Kitamura,S., Sato,C., Ando,S., Tanabe,K., Kikuchi,K., Tsuiki,S. and Nishi,Y. (1990) *J. Biol. Chem.* **265**, 4722-4729

Ingebritsen,T.S. and Cohen,P. (1983) *Eur. J. Biochem.* **132**, 255-261

Ingebritsen,T.S., Stewart,A.A. and Cohen,P. (1983) *Eur. J. Biochem.* **132**, 297-307

Ingebritsen,T.S., Foulkes,J.G. and Cohen,P. (1983) *Eur. J. Biochem.* **132**, 263-274

Ip,W., Hartzer,M.K., Pang,Y.-Y.S. and Robson,R.M. (1985) *J. Mol. Biol.* **183**, 365-375

Ishihara,H., Martin,B.L., Brautigan,D.L., Karaki,H., Ozaki,H., Kato,Y., Fusetani,N., Watabe,S., Hashimoto,K., Uemura,D. and Hartshorne,D.J. (1989) *Biochem. Biophys. Res. Comm.* **159**, 871-877

Ito,A., Hashimoto,T., Hirai,M., Takeda,T., Shuntoh,H., Kuno,T. and Tanaka,C. (1989) *Biochem. Biophys. Res. Comm.* **163**, 1492-1497

Jakes,S., Mellgren,R.L. and Schlender,K.K. (1986) *Biochim. Biophys. Acta* **888**, 135-142

Jessus,C., Goris,J., Staquet,S., Cayla,X., Ozon,R. and Merlevede,W. (1989) *Biochem. J.* **260**, 45-51

Johnson,G.V.W. and Jope,R.S. (1988) *Brain Res.* **456**, 95-103

Jones,J.C.R., Goldman,A.E., Yang,H.-Y. and Goldman,R. (1985) *J. Cell Biol.* **100**, 93-102

Jones,S.M. and Williams,R.C.,Jr. (1982) *J. Biol. Chem.* **257**, 9902-9905

Joshi,H.C. and Cleveland,D.W. (1988) *J. Cell Biol.* **109**, 663-673

Joshi,H.C., Yen,T.J. and Cleveland,D.W. (1987) *J. Cell Biol.* **105**, 2179-2190

Julien,J.-P. and Mushynski,W.E. (1981) *J. Neurochem.* **37**, 1579-1585

Julien,J.-P. and Mushynski,W.E. (1982) *J. Biol. Chem.* **257**, 10,467-10,470

Julien,J.-P. and Mushynski,W.E. (1983) *J. Biol. Chem.* **258**, 4019-4025

Julien,J.-P., Smoluk,G.D. and Mushynski,W.E. (1983) *Biochim. Biophys. Acta* **755**, 25-31

Julien,J.-P., Meyer,D., Flavell,D., Hurst,J. and Grosveld,F. (1986) *Mol. Brain Res.* **1**, 243-250

Julien,J.-P., Grosveld,F., Yazdanbaksh,K., Flavell,D., Meijer,D. and Mushynski,W.E. (1987) *Biochem. Biophys. Acta* **909**, 10-20

Julien,J.-P., Côté,F., Beaudet,L., Sidky,M., Flavell,D., Grosveld,F. and Mushynski,W.E. (1988) *Gene* **68**, 307-314

Jurgensen,S., Schacter,E., Huang,C., Chock,P.B. and Yang,S.D. (1984) *J. Biol. Chem.* **259**, 5864-5870

Kanai,Y., Takemura,R., Ohshima,T., Mori,H., Ihara,Y., Yanagisawa,M., Masaki,T. and Hirokawa,N. (1989) *J. Cell Biol.* **109**, 1173-1184

Kateo,Y., Fusetani,N., Matsunaga,S. and Hashimoto,K. (1986) *J. Am. Chem. Soc.*

108, 2780-2781

Kaufmann,E., Geisler,N. and Weber,K. (1984) *FEBS Lett.* **170**, 81-84

Kelly,B.M., Gillespie,C.S., Sherman,D.L. and Brophy,P.J. (1992) *J. Cell Biol.* **118**, 397-410

Kilmartin,J.V., Wright,B. and Milstein,C. (1982) *J. Cell Biol.* **93**, 576-582

King,,M.M., Huang,C.Y., Chock,P.B., Nairn,A.C., Hemmings,H.C.Jr., Jesse Chan,K.F. and Greengard,P. (1984) *J. Biol. Chem.* **259**, 8080-8083

Kitagawa,Y., Tahira,T., Ikeda,I., Kikuchi,K., Tsuiki,S., Sugimura,T. and Nagao,M. (1988) *Biochim. Biophys. Acta* **951**, 123-129

Klee,C.B. and Cohen,P. (1988) *Mol. Asp. Cell Reg.* **5**, 225-248

Klumpp,S., Cohen,P. and Schultz,J.E. (1990) *EMBO J.* **9**, 685-689

Klymkowsky,M.W., Maynell,L.A. and Nislow,C. (1991) *J. Cell Biol.* **114**, 787-797

Kobayashi,M., Kato,K. and Sato,S. (1975) *Biochim. Biophys. Acta* **377**, 343-355

Kobayashi,S., Ishiguro,K., Omori,A., Takamatsu,M. Arioka,M., Imahori,K. and Uchida,T. (1993) *FEBS Lett.* **335**, 171-175

Kobayashi,T., Kanno,S., Terasawa,T., Murakami,T., Ohnishi,M., Ohtsuki,K., Hiraga,A. and Tamura,S. (1993) *Biochem. Biophys. Res. Comm.* **195**, 484-489

Koonce,M.P. and Schliwa,M. (1985) *J. Cell Biol.* **100**, 322-326

Korn,E.D. (1982) *Physiol. Rev.* **62**, 672-737

Kreis,T.E. (1987) *EMBO J.* **6**, 2597-2606

Kuret,J., Bell,H. and Cohen,P. (1986) *FEBS Lett.* **203**, 197-202

Lasek,R.J., Oblinger,M. and Drake,P. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **18**, 731-744

Lazarides,E. and Woods,C. (1989) *Annu. Rev. Cell Biol.* **5**, 427-452

Ledbetter,M.C. and Porter,K.R. (1964) *Science* **144**, 872-874

Lee,M.G., Lewis,S.A., Wilde,C.D. and Cowan,N.J. (1983) *Cell* **33**, 477-487

Lee,M.G., Lewis,S.A. and Cowan,N.J. (1984) *Nucl. Acid Res.* **12**, 5823-5836

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

Lee,V.M.-Y., Otvos,L.Jr., Carden,M.J., Hollosi,M., Dietzschold,B. and Lazzarini,R.A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1998-2002

Lees,J.F., Shneidman,P.S., Skuntz,S.F., Carden,M.J. and Lazzarini,R.A. (1988) *EMBO J.* **7**, 1947-1955

Lefebvre,S. and Mushynski,W.E. (1987) *Biochem. Biophys. Res. Comm.* **145**, 1006-1011

Letai,A., Coulombe,P.A. and Fuchs,E. (1992) *J. Cell Biol.* **116**, 1181-1195

Leterrier,J.-F., Liem,R.K.H. and Shelanski,M.L. (1982) *J. Cell Biol.* **95**, 982-986

Levy,E., Liem,R.K.H., D'Eustachio,P. and Cowan,N.J. (1987) *Eur. J. Biochem.* **166**, 71-77

Lew,J., Winkfein,R.J., Paudel,H.K and Wang,J.H. (1992) *J. Biol. Chem.* **267**, 25,922-25,926

Lewis,S.A. and Cowan,N.J. (1985) *J. Cell Biol.* **100**, 843-850

Lewis,S.A., Lee,M.G. and Cowan,N.J. (1985) *J. Cell Biol.* **101**, 852-861

L'Hernault,S.W. and Rosenbaum,J.L. (1983) *J. Cell Biol.* **97**, 258-269

L'Hernault,S.W. and Rosenbaum,J.L. (1985a) *Biochemistry* **24**, 473-478

L'Hernault,S.W. and Rosenbaum,J.L. (1985b) *J. Cell Biol.* **100**, 457-462

Li,H.C., Price,D.J. and Tabarini,D. (1985) *J. Biol. Chem.* **260**, 6416-6426

Link,W.T., Doseméci,A., Floyd,C.C. and Pant,H.C. (1993) *Neurosci. Lett.* **151**, 89-93

Liu,Y. and Storm,D.R. (1989) *J. Biol. Chem.* **264**, 12,800-12,804

Ludeña,R.F., Roach,M.C., Jordan,M.A. and Murphy,D.B. (1985) *J. Biol. Chem.* **260**, 1257-1264

MacDougall,L.K., Campbell,D.G., Hubbard,M.J. and Cohen,P. (1989) *Biochim. Biophys. Acta* **1010**, 218-226

MacDougall,L.K., Jones,L.R. and Cohen,P. (1991) *Eur. J. Biochem.* **196**, 725-734

MacKintosh,C. and Klumpp,S. (1990) *FEBS Lett.* **277**, 137-140

Maro,B., Sauron,M.-E., Paulin,D. and Bornens,M. (1983) *Biol. Cell* **47**, 243-246

Matus,A. (1988) *Annu. Rev. Neurosci.* **11**, 29-44

Mayer,R.E., Hendrix,P., Cron,P., Matthies,R., Stone,S.R., Goris,J., Merlevede,W., Hofsteenge,J. and Hemmings,B.A. (1991) *Biochemistry* **30**, 3589-3597

McGowan,C.H. and Cohen,P. (1987) *Eur, J. Biochem.* **166**, 713-721

Meister,B., Fried,G., Hokfelt,T., Hemmings,H.C.Jr. and Greengard,P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8713-8716

Merdes,A., Gounari,F. and Georgatos,S.D. (1993) *J. Cell Biol.* **123**, 1507-1516

Merlevede,W. and Riley,G.A. (1966) *J. Biol. Chem.* **241**, 3517-3524

Minami,Y. and Sakai,H. (1985) *FEBS Lett.* **185**, 239-242

Mitchison,T.J. and Kirschner,M. (1984) *Nature* **312**, 237-242

Mivechi,N.F., Trainor,L.D. and Hahn,G.M. (1993) *Biochem. Biophys. Res. Comm.* **192**, 954-963

Miyasaka,H., Okabe,S., Ishiguro,K., Uchida,T. and Hirokawa,N. (1993) *J. Biol. Chem.* **268**, 22,695-22,702

Miyata,Y., Hoshi,M., Nishida,E., Minami,Y. and Sakai,H. (1986) *J. Biol. Chem.* **261**, 13,026-13,030

Mizutani,M., Nunoya,T., Yamasaki,H. and Itakura,C. (1992) *J. Hered.* **83**, 234-235

Mulligan,L., Balin,B.J., Lee,V.M.-Y. and Ip,W. (1991) *J. Struct. Biol.* **106**, 145-160

Mumby,M.C., Russell,K.L., Garrard,L.J. and Green,D.D. (1987) *J. Biol. Chem.* **262**, 6257-6265

Muñoz,D.G., Greene,C., Peel,D.P. and Selkoe,D.J. (1988) *J. Neuropathol. Exp. Neurol.* **47**, 9-18

Murthy,A.S. and Flavin,M. (1983) *Eur. J. Biochem.* **137**, 37-46

Myers,M.W., Lazzarini,R.A., Lee,V.M.-Y., Schlaepfer,W.W. and Nelson,D.L. (1987) *EMBO J.* **6**, 1617-1626

Nakamura,Y., Takeda,M., Aimoto,S., Hariguchi,S., Kitajima,S. and Nishimura,T. (1993) *Eur. J. Biochem.* **272**, 565-571

Nakamura,Y., Takeda,M., Angelides,K.J., Tanaka,T., Tada,K. and Nishimura,T. (1990) *Biochem. Biophys. Res. Comm.* **169**, 744-750

Napolitano,E.W., Chin,S.S.M., Colman,D.R. and Liem,R.K.H. (1987) *J. Neurosci.* **7**, 2590-2599

Nash,J.A.B. and Carden,M.J. (1991) *Biochem. Soc. Trans.* **19**, 1147-1148

Nemenoff,R.A., Blackshear,P.J. and Avruch,J. (1983) *J. Biol. Chem.* **258**, 9437-9443

Nimmo,G.A. and Cohen,P. (1978) *Eur. J. Biochem.* **87**, 341-351

Nishida,E., Kuwaki,T. and Sakai,H. (1981) *J. Biochem.* **90**, 575-578

Nishizawa,K., Yano,T., Shibata,M., Ando,S., Saga,S., Takahashi,T. and Inagaki,M. (1991) *J. Biol. Chem.* **266**, 3074-3079

Nixon,R.A. and Shea,T.B. (1992) *Cell Motil. Cytoskel.* **22**, 81-91

Nixon,R.A. and Sihag,R.K. (1991) *Trends Neurosci.* **14**, 501-506

Nixon,R.A., Lewis,S.E., Dahl,D., Marotta,C.A. and Drager,U.C. (1989) *Mol. Brain Res.* **5**, 93-108

Oblinger,M.M., Szumlas,R.A., Wong,J. and Liuzzi,F.J. (1989) *J. Neurosci.* **9**, 2645-2653

Ohara,O., Gahara,Y., Miyake,T., Teraoka,H. and Kitamura,T. (1993) *J. Cell Biol.* **121**, 387-395

Ohkura,H., Kinoshita,N., Miyatani,S., Toda,T. and Yanagida,M. (1989) *Cell* **57**, 997-1007

Okabe,S., Miyasaka,H. and Hirokawa,N. (1993) *J. Cell Biol.* **121**, 375-386

Olivier,A.R., Ballou,L.M. and Thomas,G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4720-4724

Otey,C.A., Kalnoski,M.H. and Bulinski,J.C. (1987) *J. Cell Biochem.* **13**, 113-124

Ottaviano,Y. and Gerace,L. (1985) *J. Biol. Chem.* **260**, 624-632

Otvos Jr.,L., Hollosi,M., Perczel,A., Dietzschold,B. and Fasman,G.D. (1988) *J. Prot. Chem.* **7**, 365-376

Pallas,D.C., Weller,W., Jaspers,S., Miller,T.B., Lane,W.S. and Roberts,T.M. (1992) *J. Virol.* **66**, 886-893

Pallen,C.J. and Wang,J.H. (1984) *J. Biol. Chem.* **259**, 6134-6141

Pant,H.C. (1988) *Biochem. J.* **256**, 665-668

Pardee,J.D. and Bamburg,J.R. (1979) *Biochemistry* **18**, 2245-2252

Parry,D.A.D. (1991) in *Cellular and Molecular Biology of Intermediate Filaments* pp. 175-204 eds. R.D. Goldman and P.M. Steinert, Plenum Press, N.Y.

Parry,D.A.D., Steven,A.C. and Steinert,P.M. (1985) *Biochem. Biophys. Res. Comm.* **127**, 1012-1018

Paschel,B.M., Shpetner,H.S. and Vallee,R.B. (1987) *J. Cell Biol.* **105**, 1273-1282

Pato,M.D. and Kerc,E. (1986) *J. Biol. Chem.* **261**, 3770-3774

Pelech,S. and Cohen,P. (1985) *Eur. J. Biochem.* **148**, 245-251

Pleasure,S.J., Lee,V.M.-Y. and Nelson,D.L. (1990) *J. Neurosci.* **10**, 2428-2437

Price,D.J. and Li,H.C. (1985) *Biochem. Biophys. Res. Comm.* **128**, 1203-1210

Price,D.J., Tabarini,D. and Li,H.C. (1986) *Eur. J. Biochem.* **158**, 635-645

Price,R.L., Lasek,R.J. and Katz,M.J. (1993) *Brain Res.* **607**, 125-133

Pruss,R.M., Mirsky,R., Raff,M.C., Thorpe,R., Dowding,A.J. and Anderton,B.H. (1981) *Cell* **27**, 419-428

Quinlan,R.A., Hatzfeld,M., Franke,W.W., Lustig,A., Schulthess,T. and Engel,J. (1986) *J. Mol. Biol.* **192**, 337-349

Redpath,N.T. and Proud,C.G. (1989) *Biochem. J.* **262**, 69-75

Reuner,K.H., Presek,P., Boschek,C.B. and Aktories,K. (1987) *Eur. J. Cell Biol.* **43**, 134-140

Roach,P.J. (1991) *J. Biol. Chem.* **266**, 14,139-14,142

Robinson,P.J. (1992) *Mol. Neurobiol.* **5**, 87-130

Roder,H.M. and Ingram,V.M. (1991) *J. Neurosci.* **11**, 3325-3343

Rosenbaum,L.C., Malencik,D.A., Anderson,S.R., Tota,M.R. and Schimerlik,M.I. (1987) *Biochemistry* **26**, 8183-8188

Rosevear,E., McReynolds,M. and Goldman,R.D. (1990) *Cell Motil. Cytoskel.* **17**, 150-166

Rossie,S. and Catterall,W.A. (1989) *Soc. Neurosci. Meeting* **15**, 514.1 (Abstr)

Rothnagel,J.A., Dominey,A.M., Dempsey,L.D., Longley,M.A., Greenhalgh,D.A., Gagne,T.A., Huber,M., Frenk,E., Hohi,D. and Roop,D.A. (1992) *Science* **257**, 1128-1130

Runge,M.S., El-Magbrabi,M.R., Clans,T.H., Pilkis,S.J. and Williams,Jr.,R.C. (1981) *Biochemistry* **20**, 175-180

Saborio,J.L. and Palmer,E. (1981) *J. Neurochem.* **36**, 1659-1669

Sacher,M.G. Athlan,E.S. and Mushynski,W.E. (1992) *Biochem. Biophys. Res. Comm.* **186**, 524-530

Sacher,M.G. Athlan,E.S. and Mushynski,W.E. (1994) *J. Biol. Chem.* **269**, 18,480-18,484

Sakaguchi,T., Okada,M., Kitamura,T. and Kawasaki,K. (1993) *Neurosci. Lett.* **153**, 65-68

Sarria,A.J., Nordeen,S.K. and Evans,R.M. (1990) *J. Cell Biol.* **111**, 553-565

Sasaki,K., Shima,H., Kitagawa,Y., Irino,S., Sugimara,T. and Nagao,M. (1990) *Jpn. J. Cancer Res.* **81**, 1272-1279

Sattilaro,R.F. (1986) *Biochem. J.* **25**, 2003-2009

Schnapp,B.J., Vale,R.D., Sheetz,M.P. and Reese,T.S. (1985) *Cell* **40**, 455-462

Schulze,E., Asai,D.J., Bulinski,J.C. and Kirschner,M. (1987) *J. Cell Biol.* **105**, 2167-2177

Selden,S.C. and Pollard,T.D. (1983) *J. Biol. Chem.* **258**, 7064-7061

Sharp,G.A., Shaw,G. and Weber,K. (1982) *Expl. Cell Res.* **137**, 403-413

Shaw,G. (1991) in *The Neuronal Cytoskeleton* pp.185-214 ed. R.D. Burgoyne, Wiley-Liss, N.Y.

Shea,T.B. (1994) *FEBS Lett.* **343**, 131-136

Shea,T.B., Majocha,R.E., Marotta,C.A. and Nixon,R.A. (1988) *Neurosci. Lett.* **92**, 291-297

Shecket,G. and Lasek,R.J. (1982) *J. Biol. Chem.* **257**, 4788-4795

Shenolikar,S. and Nairn,A.C. (1991) *Adv. Second Messengers Phosphoprotein Res.* **23**, 1-121

Sherma,R.K., Desai,R., Waisman,D.M. and Wang,J.H. (1979) *J. Biol. Chem.* **254**, 4276-4280

Sherwin,T., Schneider,A., Sasse,R.,Seebeck,T. and Gull,K. (1987) *J. Cell Biol.* **104**, 439-446

Shetty,K.T., Veeranna and Guru,S.C. (1992) *Nerosci. Lett.* **137**, 83-86

Shetty,K.T., Link,W.T. and Pant,H.C. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6844-6848

Shima,H., Haneji,T., Hatano,Y., Kasugai,I., Sugimura,T. and Nagao,M. (1993) *Biochem. Biophys. Res. Comm.* **194**, 930-937

Sibley,D.R., Strasser,R.H., Benovic,J.L., Daniel,K. and Lefkowitz,R.J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9408-9412

Sihag,R.K., Jeng,A.Y. and Nixon,R.A. (1988) *FEBS Lett.* **233**, 181-185

Sihag,R.K. and Nixon,R.A. (1989) *J. Biol. Chem.* **264**, 457-464

Sihag,R.K. and Nixon,R.A. (1990) *J. Biol. Chem.* **265**, 4166-4171

Sihag,R.K. and Nixon,R.A. (1991) *J. Biol. Chem.* **266**, 18,861-18,867

Sim,A.T.R., Dunkley,P.R., Jarvie,P.E. and Rostas,J.A.P. (1991) *Neurosci. Lett.* **126**, 203-206

Sim,A.T.R., Ratcliffe,E., Mumby,M.C., Villa-Moruzzi,E. and Rostas,J.A.P. (1994) *J. Neurochem.* **62**, 1552-1559

Skalli,O., Chou,Y.-H. and Goldman,R.D. (1992) *Trends Cell Biol.* **2**, 308-312

Soifer,D., Nicoletti,V., Cabane,K., Mack,K. and Poulos,B. (1991) *J. Neurosci. Res.* **30**, 63-71

Sola,M.M., Langan,T. and Cohen,P. (1991) *Biochim. Biophys. Acta* **1094**, 211-216

Solomon,L.R. and Rubenstein,P.A. (1985) *J. Biol. Chem.* **260**, 7659-7664

Soppet,D.R., Beasley,L.L. and Willard,M.B. (1991) *J. Neurosci. Res.* **30**, 42-46

Steinert,P.M. and Roop,D. (1988) *Annu. Rev. Biochem.* **57**, 575-609

Sternberger,L.A. and Sternberger,N.H. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6126-6130

Sternberger,N.H., Sternberger,L.A. and Ulrich,J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4274-4276

Steven,A.C., Hainfield,J.F., Trus,B., Wall,J.S. and Steinert,P.M. (1983) *J. Cell Biol.* **97**, 1939-1944

Stewart,A.A., Ingebritsen,T.S. and Cohen,P. (1983) *Eur. J. Biochem.* **132**, 289-295

Stewart,M., Quinlan,R.A. and Moir,R.D. (1989) *J. Cell Biol.* **109**, 225-234

Stone,S.R., Hofsteenge,J. and Hemmings,B.A. (1987) *Biochemistry* **26**, 7215-7220

Stossel,T.P., Chaponnier,C., Ezzell,R.M., Hartwig,J.H., Janmey,P.A., Kwiatkowski,D.J., Lind,L.E., Smith,D.B., Southwick,F.S., Yin,H.L. and Zaner,K.S. (1985) *Annu. Rev. Cell Biol.* **1**, 353-402

Strålfors,P., Hiraga,A. and Cohen,P. (1985) *Eur. J. Biochem.* **149**, 295-303

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

Sturgill,T.W., Ray,L.B., Erikson,E. and Maller,J.L. (1988) *Nature* **334**, 715-718

Sullivan,K.F. (1988) *Annu. Rev. Cell Biol.* **4**, 687-716

Sullivan,K.F. and Cleveland,D.W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4327-4331

Tallant,E.A. and Cheung,W.Y. (1984) *Biochemistry* **23**, 973-979

Tamura,S. and Tsuiki,S. (1980) *Eur. J. Biochem.* **111**, 217-224

Thoen,C., Van Hove,L. and Slegers,H. (1987) *Eur. J. Biochem.* **163**, 503-511

Tilney,L.G., Bryan,J., Bush,D.J., Fujiwara,K., Mooseker,M.S., Murphy,D.B. and Snyder,D.H. (1973) *J. Cell Biol.* **50**, 267-275

Tokutake,S., Liem,R.K.H. and Shelanski,M.L. (1984) *Biomed. Res.* **5**, 235-238

Tokutake,S. (1989) *Int. J. Biochem.* **22**, 1-6

Tokutake,S. and Tanaka,K. (1989) *J. Biochem.* **105**, 39-43

Toru-Delbauffe,D. and Pierre,M. (1983) *FEBS Lett.* **162**, 230-234

Toru-Delbauffe,D., Pierre,M., Osty,J., Chantoux,F. and Francon,J. (1986) *Biochem. J.* **235**, 283-289

Trifaró,J.M. (1982) *Trends Pharmac. Sci.* **3**, 389-392

Tsuiki,S., Kikuchi,K., Tamura,S., Hiraga,A. and Shneha,R. (1985) *Adv. Protein*

Phosphatases **1**, 193-214

Tung,H.Y. and Cohen,P. (1984) *Eur. J. Biochem.* **145**, 57-64

Usui,H., Imazu,M., Maeta,K., Tsukamoto,H., Azuma,K. and Takeda,M. (1988) *J. Biol. Chem.* **263**, 3752-3761

Vale,R.D., Schnapp,B.J., Reese,T.S. and Sheetz,M.P. (1985a) *Cell* **40**, 449-454

Vale,R.D., Reese,T.S. and Sheetz,M.P. (1985b) *Cell* **42**, 39-50

Vallano,M.L., Buckholz,T.M. and DeLorenzo,R.J. (1985) *Biochem. Biophys. Res. Comm.* **130**, 957-963

Van Eynde,A., Beullens,M., Stalmans,W. and Bollen,M. (1994) *Biochem. J.* **297**, 447-449

Vandekerchove,J. and Weber,K. (1984) *J. Mol. Biol.* **179**, 391-413

Vandenheede,J.R., Vanden Abeele,C.C. and Merlevede,W. (1980) *Biochem. Biophys. Res. Comm.* **136**, 16-21

Vandenheede,J.R., Yang,S.D. and Merlevede,W. (1983) *Biochem. Biophys. Res. Comm.* **115**, 871-876

Vikstrom,K.L., Borisy,G.G. and Goldman,R.D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 549-553

Villa-Moruzzi,E. and Heilmeyer Jr.,L.M.G. (1986) *Adv. Prot. Phosphatases* **3**, 225-

Villa-Moruzzi,E. (1992) *FERS Lett.* **304**, 211-215

Virshup,D.M. and Kelly,T.J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3584-3588

Villasante,A., Wang,D., Dobner,P., Dolph,P., Lewis,S.A. and Cowan,N.J. (1986) *Mol. Cell Biol.* **6**, 2409-2419

Wadzinski,B.E., Wheat,W.H., Jaspers,S., Peruski Jr., L.F., Lickteig,R.L., Johnson,G.L. and Klemm,D.J. (1993) *Mol. Cell. Biol.* **13**, 2822-2834

Waelkens,E., Agostinis,P., Goris,J. and Merlevede,W. (1987) *Adv. Enzyme Regul.* **26**, 241-270

Walter,G., Ferre,F., Espiritu,O. and Carbone-Wiley,A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8669-8672

Wang,D., Villasante,A. Lewis,S.A. and Cowan,N.J. (1986) *J. Cell Biol.* **103**, 1903-1910

Wang,L.-Y., Orser,B.A., Brautigan,D.L. and MacDonald,J.F. (1994) *Nature* **369**, 230-232

Wang,S., Hamberger,A., Ding,M. and Haglid,K.G. (1992) *J. Neurochem.* **59**, 1975-1978

Wang,S., Hamberger,A., Yang,Q. and Haglid,K.G. (1994) *J. Neurochem.* **62**, 739-748

Wehland,J., Schroder,H.C. and Weber,K. (1984) *EMBO J.* 3, 1295-1300

Wehland,J. and Weber,K. (1987) *J. Cell Sci.* 88, 185-203

Wenk,J., Trompeter,H.-I., Pettrich,K.-G., Cohen,P.T.W., Campbell,D.G. and Mieskes,G. (1992) *FEBS Lett.* 297, 135-138

Wera,S. Bollen,M. and Stalmans,W. (1991) *J. Biol. Chem.* 266, 339-345

Wible,B.A., Smith,K.E. and Angelides,K.J. (1989) *Proc. Natl. Acad. Sci. USA* 86, 720-724

Wiley,C.A., Love,S., Skoglund,R.R. and Lampert,P.W. (1987) *Acta Neuropathol.* 72, 369-376

Williams,K.R., Hemmings,H.C.Jr., LoPresti,M.B., Konigsberg,W.H. and Greengard,P. (1986) *J. Biol. Chem.* 261, 1890-1903

Winkler,M.A., Merat,D.L., Tallant,E.A., Hawkins,S. and Cheung,W.Y. (1984) *Proc. Natl. Acad. Sci. USA* 81, 3054-3058

Wong,P.C., and Cleveland,D.W. (1990) *J. Cell Biol.* 111, 1987-2003

Xiao,J. and Monteiro,M.J. (1994) *J. Neurosci.* 14, 1820-1833

Xu,Z., Cork,L.C., Griffin,J.W. and Cleveland,D.W. (1993) *Cell* 73, 23-33

Xu,Z.-S., Liu,W.-S. and Willard,M.B. (1990) *J. Neurosci.* 10, 1838-1846

Xu,Z.-S., Liu,W.-S. and Willard,M.B. (1992) *J. Biol. Chem.* **267**, 4467-4471

Yamamoto,H., Saitoh,Y., Fukunaga,K., Nishimura,H. and Miyamoto,E. (1988) *J. Neurochem.* **50**, 1614-1623

Yamamoto,H., Saitoh,Y., Yusugawa,S. and Miyamoto,E. (1990) *J. Neurochem.* **55**, 683-690

Yamasaki,H., Itakura,C. and Mizutani,M. (1991) *Acta Neuropathol.* **82**, 427-434

Yamasaki,H., Bennett,G.S., Itakura,C. and Mizutani,M. (1992) *Lab. Invest.* **66**, 734-743

Yang,H.-Y., Lieska,N. and Goldman,R.D. (1991) in *Cellular and Molecular Biology of Intermediate Filaments* pp.371-387 eds. R.D. Goldman and P.M. Steinert, Plenum Presss, N.Y.

Yang,S.D., Vandenheede,J.R.. and Merlevede,W. (1981) *J. Biol. Chem.* **256**, 10,231-10,234

Yang,S.D., Vandenheede,J.R.. and Merlevede,W. (1983) *Biochem. Biophys. Res. Comm.* **113**, 439-445

Zhang,Z., Bai,G., Shima,M., Zhao,S., Nagao,M. and Lee,E.Y.C. (1993) *Arch. Biochem. Biophys.* **303**, 402-406

Zimmerman,U.-J.P. and Schlaepfer,W.W. (1986) *Biochemistry* **25**, 3533-3536

Chapter 2

OKADAIC ACID INDUCES THE RAPID AND REVERSIBLE DISRUPTION OF THE NEUROFILAMENT NETWORK IN RAT DORSAL ROOT GANGLION NEURONS

Summary

Treatment of dissociated cultures of rat dorsal root ganglia with 1 μM okadaic acid caused a reduction in the mobilities of neurofilament subunits on SDS-polyacrylamide gels, signifying an increase in their phosphorylation levels. When cultures were exposed to okadaic acid for 0.5 hrs. and harvested in buffer containing Triton X-100, NF-H was nearly completely redistributed to the detergent- soluble fraction while NF-M and NF-L required a longer exposure to the drug before undergoing a similar shift. This redistribution of subunits corresponded with striking changes in the immunofluorescence staining pattern for neurofilaments. Upon removal of okadaic acid from the culture medium following a 0.5 hr. treatment, NF-L and NF-M returned to the Triton X-100 insoluble fraction within 2 hrs. while NF-H required 10 hrs. for recovery.

Introduction

Neurofilaments (NFs) belong to the class of cytoskeletal proteins known as intermediate filaments and are expressed specifically in neurons. Mammalian NFs consist of three phosphoprotein subunits, NF-H, NF-M and NF-L, whose apparent molecular weights on SDS-polyacrylamide gels (SDS-PAGE) are 200 kDa, 145 kDa and 68 kDa, respectively, for the highly phosphorylated forms (Hoffman and Lasek, 1975; Julien and Mushynski, 1982). The role of phosphorylation in NF function remains unclear, although it has been implicated in a variety of processes (Minami and Sakai, 1985; Goldstein *et al*, 1987; Pant, 1988; Nakamura *et al*, 1990; Hisanaga *et al*, 1990; Gonda *et al*, 1990). Since phosphate moieties undergo turnover as NFs are transported down the axon (Nixon *et al*, 1987) it appears that some aspect(s) of NF metabolism may be modulated by cyclical phosphorylation and dephosphorylation events.

Okadaic acid (OA) is a complex fatty acid derivative found in dinoflagellates which specifically inhibits protein phosphatase 1 (PP-1) and protein phosphatase 2A (PP-2A) with IC_{50} values of 10 nM and 0.1 nM, respectively (Bialojan and Takai, 1988). The effects of this tumour promotor on different cell types is varied (see Hardie *et al*, 1991, for review). In fibroblasts, OA has recently been shown to increase phosphorylation of another intermediate filament protein, vimentin (Yatsunami *et al*, 1991). We therefore set out to determine what effect OA would have on the three NF subunits in the neurons of cultured rat dorsal root ganglia (DRG). We show that OA caused a disruption of the NF network characterized by the hyperphosphorylation of NF subunits and disassembly of NFs. These effects were reversible within 10 hrs. after removal of OA.

Materials and Methods

Materials: OA was purchased from LC Services Corp. (Woburn, MA). Insulin, progesterone, selenium, putrescine and all monoclonal antibodies (antiNF-H, N52; antiNF-M, NN18; antiNF-L, NR4) were purchased from Sigma Chemical Co. (St. Louis, MO). Transferrin was from ICN, Canada.

Cell Culture: Rat DRG were dissected from E15 embryos, dissociated with trypsin (Wood and Bunge, 1986) and maintained in defined medium (Bottenstein and Sato, 1979). Where indicated, Triton buffer contained 1% Triton X-100, 50 mM TrisHCl pH 7.5, 100 mM NaCl, 2 mM EGTA, 2 mM levamisole, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 25 μM leupeptin and 40 U/mL aprotinin.

Gel Electrophoresis and Western Blotting: Proteins were solubilized in SDS sample buffer and fractionated on 5% SDS-polyacrylamide slab gels (Laemmli, 1970). Proteins were electrophoretically transferred to Immobilon-P membranes (Millipore Corp.) in buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. The membrane was blocked with 0.5% gelatin in TBS-Tween (20 mM TrisHCl pH 7.6, 137 mM NaCl and 0.1% Tween-20), incubated with primary antibody and detected using the ECL Western Blotting Detection Kit (Amersham) following the manufacturers instructions. Two-dimensional gel electrophoresis was performed by the method of O'Farrell *et al.* (1977).

Immunofluorescence microscopy: Cells were rinsed, fixed with ethanol/acetic acid (90:10), blocked with goat serum for 1 hr., incubated with primary antibody for 1 hr., washed and incubated with antimouse-Texas Red conjugated antibody for 1 hr. and mounted on coverslips.

Results

To begin elucidating the role of phosphorylation in NF organization and distribution we chose to treat rat DRG with OA, an inhibitor of PP-1 and PP-2A. Various concentrations of OA were tested for their effects on the behaviour of the three NF subunits on SDS-PAGE as increased phosphorylation of NF-M and NF-H is known to reduce their mobilities (Julien and Mushynski, 1982). The least phosphorylated form of NF-H did not shift noticeably until 0.1μM OA and continued to increase in apparent molecular weight at OA concentrations up to 1 μM (data not shown). Slower migrating forms of NF-L and NF-M were seen at a concentration of 0.5μM OA and persisted at 1 μM OA. We chose to use 1 μM OA for all subsequent experiments, as this concentration is recommended for intact cells (Haystead *et al*, 1989; Hardie *et al*, 1991; Hernandez-Sotomayor *et al*, 1991).

Fig.1 shows the distribution of the three NF subunits in Triton-soluble (S) and insoluble (P) fractions. After treatment with OA, cells were harvested in buffer containing Triton and centrifuged at 15,000xg for 20 minutes. In control cells all three subunits were found in the Triton-insoluble pellet fraction, signifying their association with the NF network. Within 0.5 hrs. of OA treatment almost all of the NF-H was redistributed to the Triton-soluble supernatant along with smaller proportions of NF-M and NF-L. By 3 hrs. all of the NF-M was redistributed to the supernatant fraction while it took 6 hrs. for NF-L to disappear completely from the pellet. An increase in the apparent molecular weights of NF-M (to pM) and NF-L (to pL) was seen after 6 hrs. of exposure to OA whereas the mobility of NF-H was seen to decrease as early as 1 hr. Furthermore, 2-dimensional gel electrophoresis showed an acidic shift in the pI values for slower migrating NF-M (pM) and NF-L (pL) consistent with their increased phosphorylation states (data not shown). Since all three NF subunits were detected in the Triton-soluble fraction within 0.5 hrs. we used immunofluorescence microscopy to determine whether this shift was accompanied by changes in the cytoskeletal organization of NFs. Although no changes in electrophoretic mobility of NF subunits were seen after 0.5 hrs. of OA treatment, there were marked changes in the immunofluorescence staining patterns

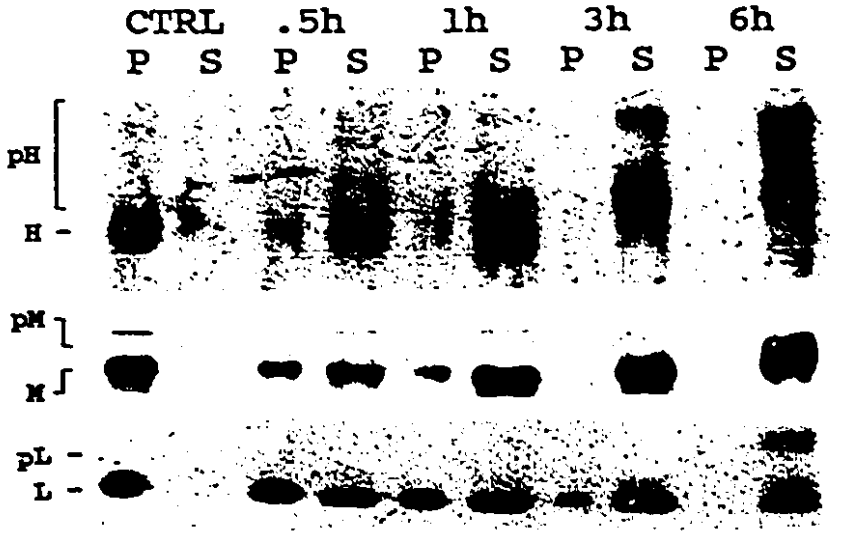

Figure 1: Western blot analysis of Triton X-100 extracts from okadaic acid-treated dorsal root ganglion cultures DRG cultures were treated with OA for the stated periods of time and extracted with buffer containing 1% Triton X-100. Pellets (P) and supernatants (S) were obtained by centrifugation, fractionated by SDS-PAGE, transferred to Immobilon-P membrane and detected using antibodies specific to the three NF subunits. H, M and L refer to the native NF subunits containing low phosphate levels while pH, pM and pL refer to the more highly phosphorylated forms of the subunits.

for all three polypeptides. A representative micrograph of cultures treated with antiNF-L is shown in Fig.2. The smooth axonal staining of control cultures differed markedly from the punctate staining seen in OA-treated samples.

In light of the rapid disruption of NFs by OA, we wished to determine whether the process was reversible after removal of OA from the culture medium. DRG cultures were treated with OA for 0.5 hrs., washed three times with OA-free medium and allowed to recover for various times in OA-free medium containing 10% horse serum and 5% fetal bovine serum. To determine the distribution of NF subunits during recovery, cells were harvested with Triton-containing buffer at various times after removal of OA and Triton-soluble (S) and insoluble (P) fractions were separated as described for Fig.1. As seen in Fig.3, NF-L and NF-M returned to the Triton-insoluble fraction within 2 hrs. while NF-H was seen in the insoluble fraction within 10 hrs. after removal of OA from the medium.

These results indicate that OA causes an increase in phosphate content of NF subunits resulting in the rapid and reversible disruption of the NF network.

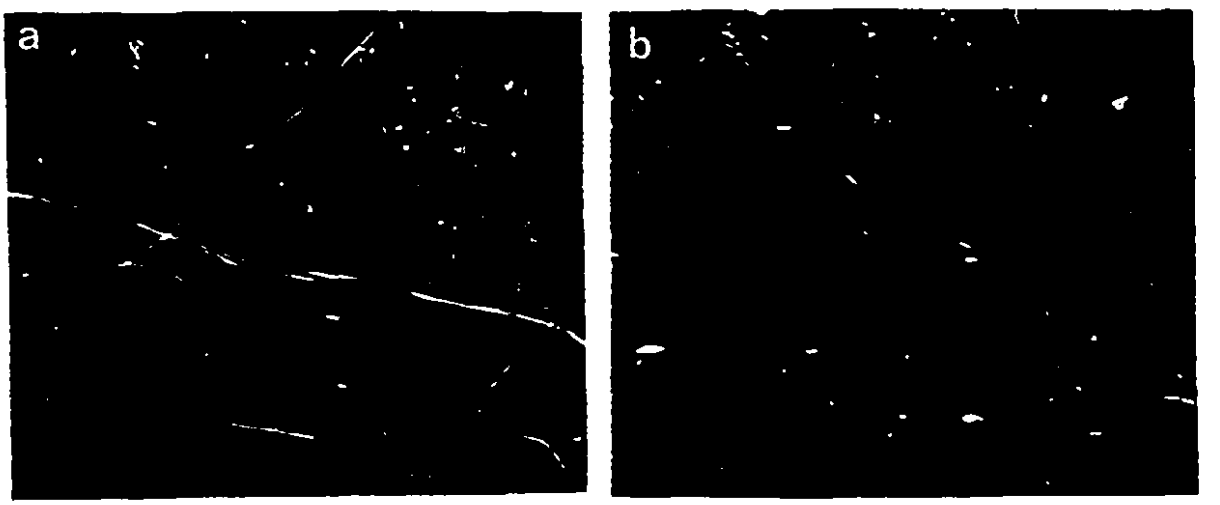

Figure 2: Immunofluorescence microscopy of control and okadaic acid-treated dorsal root ganglion cultures DRG cultures were untreated (a) or treated with OA (b) for 0.5 hrs., fixed and stained with an antiNF-L monoclonal antibody followed by an antimouse IgG conjugated to Texas Red.

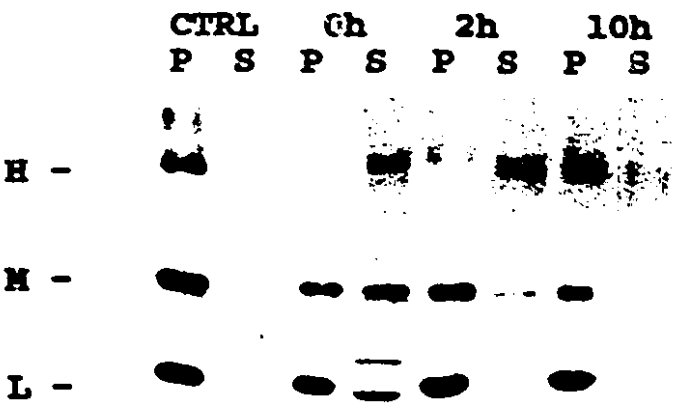

Figure 3: Western blot analysis of dorsal root ganglion cultures recovering from okadaic acid treatment DRG cultures were treated with 1 μM OA for 0.5 hrs., washed and grown in OA-free medium containing serum for the times shown. Cells were separated into Triton X-100 soluble (S) and insoluble (P) fractions for immunoblot analysis using antibodies specific for the three NF subunits. The NF subunits are designated as described in the legend to Fig. 1.

Discussion

The present results indicate that phosphorylation plays a major role in the dynamics of NF organization. The sequential appearance of NF subunits in the Triton-soluble fraction (Fig.1) follows the order expected from one of the proposed schemes of NF organization in which NF-L is suggested to form the filament core while NF-M and NF-H have a more peripheral association (Sharp *et al*, 1982; Tokutake, 1990). If the NFs were merely being fragmented in the presence of OA one would expect them to appear in the Triton-soluble fraction simultaneously rather than in the order seen in Fig.1. This result also suggests that the subunits are incorporated as homo- rather than heterooligomeric units during NF assembly. Fig.3 shows that the effects of short term OA treatment of DRG cultures can be reversed, with signs of recovery being apparent as early as 2 hrs. post-treatment for NF-L and NF-M and 10 hrs. for NF-H. The order of NF subunit reappearance in the Triton-insoluble fraction again lends support to the notion of an ordered assembly of homooligomeric units. In light of the extensive nature of the NF network in DRG neurons it is likely that a large proportion of the original subunits are reincorporated into the filamentous network.

The punctate immunofluorescence staining pattern seen in OA-treated cells is intriguing considering that much of NF-L and NF-M still remained in the Triton-insoluble fraction at 0.5 hrs. (Fig.1). This feature was seen even with antiNF-M suggesting that solubilization of NF-H and/or increased subunit phosphorylation disrupts the NF network.

Tryptic phosphopeptide mapping of NF subunits labelled with ^{32}P in the presence of OA may show which phosphorylated region is responsible for NF disassembly. Previous studies on other intermediate filaments, as well as on NF-L, have shown that *in vitro* phosphorylation of the amino terminal head domain disrupts the filaments and prevents their reassembly (Geisler and Weber, 1988; Ando *et al*, 1989; Gonda *et al*, 1990). Furthermore, protein kinase A has recently been shown to phosphorylate the head domain of NF-M (Sihag and Nixon, 1990),

although its effect on NF-M assembly has not yet been studied. Whether OA-induced disassembly of the NFs *in vivo* is due to head domain phosphorylation remains to be determined.

A decreased mobility on SDS-PAGE was seen for all three NF subunits (Fig.1). The appearance of a second form of NF-L (pL) was unexpected as only the 68 kDa species has been reported to date. This shift in mobility was due to phosphorylation of the protein as the pI for the slower migrating form was more acidic than that of the native form (data not shown). The lack of intermediate forms between the two NF-L species (L and pL in Fig.1) may indicate that the shift in mobility is due to one specific phosphorylation event rather than the end result of a series of phosphorylations. While phosphorylation variants of NF-M are well known (Georges *et al*, 1989), the physiological relevance of the slower migrating form of NF-L is unknown.

It is not clear whether the increased phosphorylation states of NF subunits in OA-treated neurons was due to the activation of protein kinase(s) and/or inhibition of NF dephosphorylation. Phosphorylated Lys-Ser-Pro sites in histone H1 analagous to the sites found in the tail domains of NF-M and NF-H have been shown to be dephosphorylated by PP-2A (Sola *et al*, 1991). It should be noted that PP-2A has also been reported to negatively regulate cdc2 kinase (Félix *et al*, 1990) and *in vitro* studies have shown this kinase to be capable of phosphorylating the repeated Lys-Ser-Pro sites in dephosphorylated NF-H (Hisanaga *et al*, 1990; Guan *et al*, 1992). Perhaps a related kinase under similar regulation is involved in phosphorylating these sites *in vivo*.

References

Ando, S., Tanabe, K., Gonda, Y., Sato, C. and Inagaki, M. (1989) *Biochemistry* **28**, 2974-2979

Bialojan, C. and Takai, A. (1988) *Biochem. J.* **256**, 283-290

Bottenstein, P.J. and Sato, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 514-517

Félix, M.-A., Cohen, P. and Karsenti, E. (1990) *EMBO J.* **9**, 675-683

Geisler, N. and Weber, K. (1988) *EMBO J.* **7**, 15-20

Georges, E., Lindenbaum, M.H., Sacher, M.G., Tifaró, J.-M. and Mushynski, W.E. (1989) *J. Neurochem.* **52**, 1156-1161

Goldstein, M., Sternberger, N.H. and Sternberger, L.A. (1987) *J. Neuroimmun.* **14**, 149-160

Gonda, Y., Nishizawa, K., Ando, S. Kitamura, S., Minoura, Y., Nishi, Y. and Inagaki, M. (1990) *Biochem. Biophys. Res. Comm.* **167**, 1316-1325

Guan, R.J., Hall, F.L. and Cohlberg, J.A. (1992) *J. Neurochem.* **58**, 1365-1371

Hardie, D.G., Haystead, T.A.J. and Sim, A.T.R. (1991) *Meth. Enzymol.* **201**, 469-476

Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P. and Hardie, D.G. (1989) *Nature* **335**, 78-81

Hernandez-Sotomayor, S.M., Mumby, M. and Carpenter, G. (1991) *J. Biol. Chem.* **266**, 21281-21286

Hisanaga, S., Gonda, Y., Inagaki, M., Ikai, A. and Hirokawa, N. (1990) *Cell Regul.* **1**, 237-248

Hisanaga, S., Kusubata, M., Okumura, E. and Kishimoto, T. (1991) *J. Biol. Chem.* **266**, 21798-21803

Hoffman, P.N. and Lasek, R.J. (1975) *J. Cell Biol.* **66**, 351-366

Julien, J.-P. and Mushynski, W.E. (1982) *J. Biol. Chem.* **257**, 10467-10470

Laemmli, U.K. (1970) *Nature* **227**, 680-685

Minami, Y. and Sakai, H. (1985) *FEBS Lett.* **185**, 239-242

Nakamura, Y., Takeda, M. Angelides, K.J., Tanaka, T., Tada, K. and Nishimura, T. (1990) *Biochem. Biophys. Res. Comm.* **169**, 744-750

Nixon, R.A., Lewis, S.E. and Marotta, C.A (1987) *J. Neurosci.* **7**, 1145-1158

O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) *Cell* **12**, 1133-1142

Pant, H.C. (1988) *Biochem. J.* **256**, 665-671

Sharp, G.A., Shaw, G. and Weber, K. (1982) *Exp. Cell Res.* **137**, 403-413

Sihag, R.K. and Nixon, R.A. (1990) *J. Biol. Chem.* **265**, 4166-4171

Sola, M.M., Langan, T. and Cohen, P. (1991) *Biochim. Biophys. Acta* **1094**, 211-216

Tokutake, S. (1990) *Int. J. Biochem.* **22**, 1-6

Wood, P.M. and Bunge, R.P. (1986) *J. Neurol. Sci.* **74**, 153-169

Yatsunami, J., Fujiki, H., Suganuma, M., Yoshizawa, S., Eriksson, J.E., Olson, M.O.J. and Goldman, R.D. (1991) *Biochem. Biophys. Res. Comm.* **177**, 1165-1170

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Chapter 3

INCREASED PHOSPHORYLATION OF THE AMINO-TERMINAL DOMAIN OF THE LOW MOLECULAR WEIGHT NEUROFILAMENT SUBUNIT IN OKADAIC ACID-TREATED NEURONS

Summary

Treatment of rat dorsal root ganglion cultures with 1 μM okadaic acid leads to a fragmentation of neurofilaments and a reduction in the electrophoretic mobilities of the three subunits on SDS-polyacrylamide gels (Sacher *et al*, 1992). Based on the observed response to varying concentrations of okadaic acid, fragmentation was inferred to be due to inhibition of protein phosphatase-2A activity and reduction in electrophoretic mobility to inhibition of protein phosphatase-1. Okadaic acid treatment led to an increase in amino-terminal, relative to carboxy-terminal, domain phosphorylation in the low molecular weight (NF-L) subunit in the Triton-soluble and -insoluble fractions. The purified catalytic subunit of protein phosphatase-2A dephosphorylated ^{32}P-labelled NF-L and the middle molecular weight subunit from okadaic acid-treated cultures while the catalytic subunit of protein phosphatase-1 had no effect. In the case of NF-L, phosphate moieties were preferentially removed from the amino-terminal domain. These results show that the amino-terminal domain of NF-L can be phosphorylated *in situ* and implicate protein phosphatase-2A in the turnover of phosphate moieties in this domain.

Introduction

Neurofilaments (NFs) are components of the cytoskeleton of most neurons and are composed of three subunits belonging to the type IV subclass of intermediate filament (IF) proteins (Steinert and Roop, 1988). The three subunits have apparent molecular weights on SDS-polyacrylamide gels of 68,000 (NF-L), 145,000 (NF-M), and 200,000 (NF-H) (Hoffman and Lasek, 1975) although the deduced molecular weights of the subunits from rats are 61,000 (NF-L; Chin and Liem, 1989), 95,000 (NF-M; Napolitano *et al*, 1987), and 115,000 (NF-H; Chin and Liem, 1990). All three subunits contain multiple phosphate moieties (Julien and Mushynski, 1982) and are rich in charged amino acid residues. Both the phosphate moieties (Julien and Mushynski, 1982) and glutamic acid residues (Georges and Mushynski, 1987) have been shown to contribute to the anomalous migration of NF subunits on SDS-polyacrylamide gels.

Much of the work to date on NF subunit phosphorylation has focussed on identifying the protein kinases responsible for phosphorylating the subunits. For example, many protein kinases have been shown to copurify with NF preparations (Julien *et al*, 1983; Toru-Delbauffe and Pierre, 1983; Vallano *et al*, 1985; Caputo *et al*, 1989; Wible *et al*, 1989; Dosemeci *et al*, 1990) and to phosphorylate NF subunits *in vitro*. Recently, cdc2-kinase from starfish oocytes was shown to phosphorylate the dephosphorylated form of NF-H, returning its mobility on SDS-gels to that of the native form (Hisanaga *et al*, 1991). Subsequently, several laboratories have identified and cloned cdc2-like protein kinases from nervous tissue (Hellmich et al, 1992; Lew *et al*, 1992; Shetty *et al*, 1993) These kinases are capable of phosphorylating peptides containing the sequence Lys-Ser-Pro, which occurs in multiple copies in both NF-M and NF-H. Furthermore, F_A-kinase, the component required for protein phosphatase-1 (PP-1) activation, was also shown to phosphorylate NFs *in vitro* (Guan *et al*, 1991). NFs were also shown to be *in vitro* (Sihag *et al*, 1988) and *in vivo* (Georges *et al*, 1989; Grant and Aunis, 1990) substrates for protein kinase C.

Conversely, very little is known about the protein phosphatases involved in maintaining the phosphorylation state of NF subunits. Shetty *et al* (1992) characterized a protein phosphatase activity which copurifies with NF preparations from both bovine and rat spinal cord and is inhibited by aluminium, vanadate, and fluoride. Another *in vitro* study, involving the four major mammalian serine/threonine protein phosphatases (see Cohen, 1989, for review), showed that none of these enzymes were capable of promoting microtubule binding to NF-H in a manner similar to that observed after NF-H is treated with either acid or alkaline phosphatase (Hisanaga *et al*, 1993). Recent studies have shown that the PP-1 and protein phosphatase-2A (PP-2A) inhibitor okadaic acid (OA; Bialojan and Takai, 1988) causes a disruption of the NF network in rat dorsal root ganglion (DRG) neurons (Sacher *et al*, 1992) and an increased deposition of NF subunits in nb2a/d1 neuroblastoma cells (Shea *et al*, 1993).

The functional role of NF phosphorylation is unclear and may depend on the location of the phosphate moieties within the subunits (see Nixon and Sihag, 1991, for review). Previous work in our laboratory has shown that treatment of rat DRG cultures with 1 μM OA causes a rapid disruption of the NF network (Sacher *et al*, 1992). Other studies have shown that phosphorylation of the amino-terminal head domain of NF-L *in vitro* causes the subunit to dissociate from a preexisting network and prevents its assembly (Nakamura *et al*, 1990; Gonda *et al*, 1990). Similarly, head domain phosphorylation of the type III IF subunits vimentin, desmin and glial fibrillary acidic protein (GFAP), has been correlated with filament breakdown *in vitro* and *in vivo* (Geisler and Weber, 1988; Geisler *et al*, 1989; Inagaki *et al*, 1990; Chou *et al*, 1991; Matsuoka *et al*, 1992). We therefore set out to determine the distribution of phosphorylation sites in NF-L subunits after OA treatment by chemical cleavage analysis. Our results show an increase in amino-terminal domain phosphorylation in NF-L following OA treatment. Inhibition of PP-2A activity was implicated in OA-induced NF fragmentation and the catalytic subunit of PP-2A was shown to be capable of removing phosphate moieties from

the amino-terminal domain of NF-L *in vitro*.

Materials and Methods

Materials: Okadaic acid and Calyculin A were purchased from LC Services (Woburn,MA). Carrier-free $^{32}P_i$ was from ICN Biomedicals (Mississauga,ON,Canada).

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE): Gel electrophoresis and Western blotting were performed as previously described (Laemmli, 1970; Sacher *et al*, 1992). Polypeptides used for chemical cleavage analysis (see below) were obtained from gels run in the presence of 0.025% thioglycolic acid to prevent protein oxidation. Protein on Western blots was quantified using a MicroVision SCSI camera followed by analysis using the Millipore Bio-Image analyzer. Radioactivity was quantified with the Fuji BAS2000 Phosphorimager.

Immunoprecipitation: Cells were harvested in cytoskeleton extraction buffer (CSK buffer) consisting of 1% Triton X-100 (Triton), 150 mM NaCl, 50 mM TrisHCl pH 7.5, 50 mM NaF, 2 mM EGTA, 2 mM levamisol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged for 15 min. at 13,000x*g*. SDS was added to the supernatant to a concentration of 0.1% followed by heating in a boiling water bath for 2 min. The pellet was suspended in 2% SDS, 50 mM TrisHCl, pH 6.8, heated for 2 min. and then the SDS was diluted to 0.1% with CSK buffer. Immunoprecipitation was carried out as described previously (Lindenbaum *et al*, 1987) using either affinity purified antibody or immune serum. *In vitro* phosphatase treatment of the immunoprecipitates was carried out prior to elution from the protein A-sepharose beads (see below).

Cell Culture: Rat DRG were dissected and maintained in defined medium as previously described (Sacher *et al*, 1992). For metabolic labelling cells were incubated with 0.5 mCi of carrier-free $^{32}P_i$ per mL P_i-free medium (Flow Laboratories, McLean, VA) for 3 hrs. prior to OA treatments.

Phosphatase treatment of immunoprecipitated proteins: For phosphatase treatment of ^{32}P-labelled immunoprecipitates, samples were incubated for 5 hrs. at 30°c, prior to elution from the protein A-sepharose beads (see above), in 65 mM TrisHCl pH 7, 1 mM $MgCl_2$, 0.5 mM EGTA, 1 mM PMSF +/- $PP\text{-}2A_c$, $PP\text{-}1_c$ (1.5 μg/mL) and OA as described in the figure legends.

Chemical cleavage of polypeptides: Immunoprecipitated ^{32}P-labelled NF-L was resolved by SDS-PAGE, located by autoradiography and excised. The gel slice was rehydrated in N-chlorosuccinimide (NCS)-buffer (1 g Urea:1 mL water:1 mL acetic acid) (Lischwe and Ochs, 1982) and incubated at room temperature for 1.5 hrs. in the same buffer containing 2 mg/mL NCS. The slices were washed extensively in water, equilibrated in SDS-PAGE sample buffer, and loaded vertically onto an SDS-12% polyacrylamide gel. Digested products were visualized by autoradiography using a Dupont Lightning-plus intensifying screen (Swanstrom and Shank, 1978) and quantified as described above.

Preparation of protein phosphatase-1 and protein phosphatase-2A: The catalytic subunits of PP-1 and PP-2A ($PP\text{-}1_c$ and $PP\text{-}2A_c$, respectively) were purified from rabbit skeletal muscle following the procedure of Cohen *et al* (1988).

Phosphorylated amino acid analysis: Immunoprecipitated NF-L and NF-M were resolved by SDS-PAGE, located by autoradiography, excised and processed as previously described (Julien and Mushynski, 1982). Unlabelled phosphoaminoacid standards were detected by ninhydrin staining (Cooper *et al*, 1983).

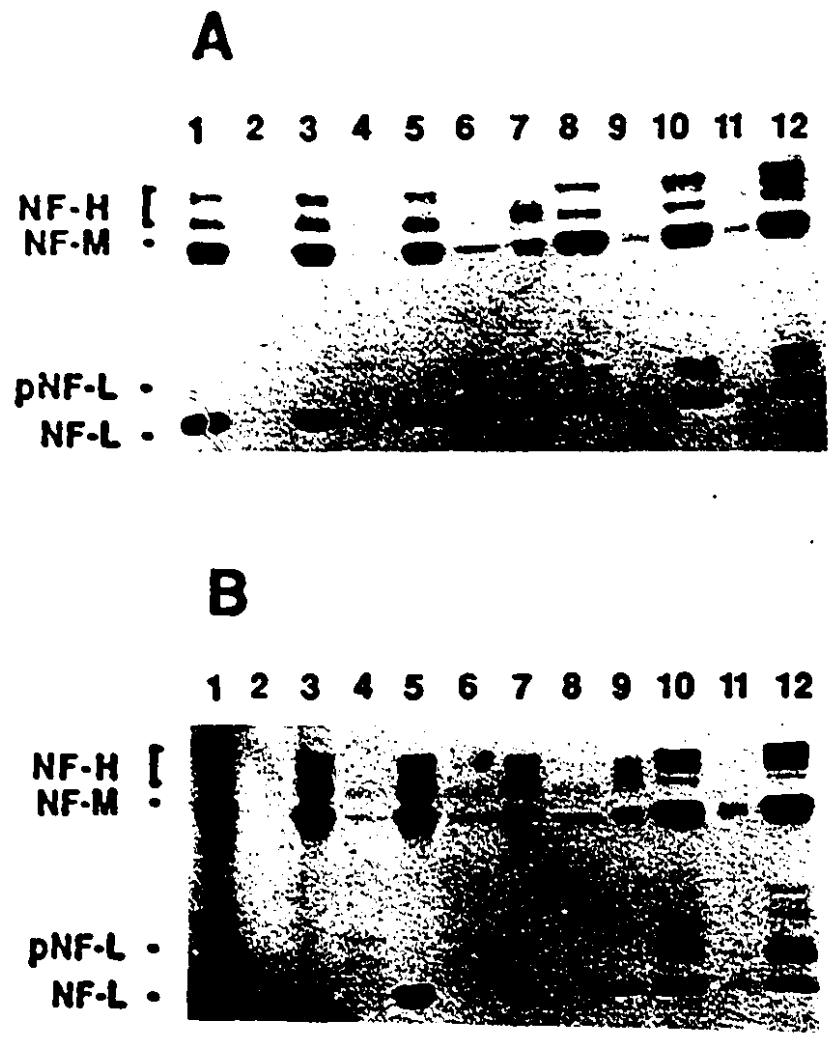
Results

To determine which phosphatase(s) plays a role in maintaining the integrity of NFs DRG cultures were treated with increasing concentrations of OA or calyculin A. OA is a more potent inhibitor of PP-2A than of PP-1 whereas calyculin A inhibits both PP-1 and PP-2A with similar potencies (Ishihara *et al*, 1989). As shown in Fig.1A, treatment of cultures with 100 nM OA for 4 hrs. led to NF fragmentation with no changes in the electrophoretic mobilities of any of the three NF subunits (Fig.1A,lanes 7 and 8). As the OA concentration was raised to 500 nM and 1000 nM fragmentation was accompanied by decreases in the electrophoretic mobilities of all three subunits (Fig.1A, compare lanes 7 and 8 with lanes 9 and 10). This would imply that fragmentation at the lower OA concentration was due to inhibition of PP-2A while the concomitant decrease in electrophoretic mobility of subunits at the higher OA concentration (Sacher *et al*,1992) was due to the additional inhibition of PP-1. This inference is supported by the results in Fig.1B showing that both NF fragmentation and decreases in subunit electrophoretic mobility occur at the same calyculin A concentration (lanes 9 and 10).

Since consensus sequences have not been established for PP-1 and PP-2A (Ingebritsen and Cohen, 1983; Agostinis *et al*, 1987) it is not possible to predict where the additional phosphate moieties would be found in the NF subunits when the phosphatases are inhibited. However, phosphoamino acid analysis shows a similar lack of phosphothreonine in NF-L and no change in the proportions of phosphoserine to phosphothreonine in NF-M after OA treatment (data not shown). These phosphoamino acid profiles correspond to those of *in vivo* ^{32}P-labelled NF-L and NF-M (Julien and Mushynski, 1982).

We examined the distribution of phosphorylation sites in NF-L by chemical cleavage with NCS (Lischwe and Ochs, 1982). Rat NF-L contains one tryptophan residue at amino acid 280 (Chin and Liem, 1989) and the two halves of the protein migrate differently on SDS-PAGE (Mahboub *et al*, 1986). Determining the ratio of carboxy-terminal to amino-terminal ^{32}P-phosphorylation (C/N ratios) provides

Figure 1: Western blot analysis of the effects of increasing OA and calyculin A concentrations on NF subunits in DRG neurons. DRG cultures were fractionated into Triton X-100-insoluble (odd numbered lanes) and -soluble fractions (even numbered lanes) after a 4 hr. exposure to either OA (A) or calyculin A (B). Samples were resolved by SDS-PAGE and the NF subunits detected by Western blotting as described in *Materials and Methods*. (A) OA concentrations used were 0 (lanes 1 and 2), 1nM (lanes 3 and 4), 10nM (lanes 5 and 6), 100nM (lanes 7 and 8), 500nM (lanes 9 and 10) and 1000nM (lanes 11 and 12). (B) Calyculin A concentrations used were 0 (lanes 1 and 2), 0.1nM (lanes 3 and 4), 0.5nM (lanes 5 and 6), 1nM (lanes 7 and 8), 10nM (lanes 9 and 10) and 100nM (lanes 11 and 12). NF-H, NF-M and NF-L refer to the positions of the three NF subunits. pNF-L refers to the slower migrating, hyperphosphorylated form of NF-L.


A
1 2 3 4 5 6 7 8 9 10 11 12
NF-H
NF-M
pNF-L
NF-L
B
1 2 3 4 5 6 7 8 9 10 11 12
NF-H
NF-M
pNF-L
NF-L

a means for assessing the location of phosphorylation sites on NF-L following OA treatment. DRG cultures were labelled with $^{32}P_i$ followed by a 1 hr. treatment with 1 μM OA. Samples were fractionated into Triton-soluble and -insoluble fractions and immunoprecipitated NF-L was treated with NCS. As shown in Fig. 2 the C/N ratio of Triton-soluble and -insoluble NF-L from OA-treated cells was reduced by about 6-fold in comparison to that of the untreated control. It is interesting to note that the C/N ratios of both the Triton-soluble and -insoluble NF-L from OA-treated cultures were the same. These results indicate that while OA treatment causes a preferential increase in amino terminal domain phosphorylation in NF-L the Triton soluble subunit shows no further increase in phosphorylation of this domain.

Although enhanced phosphorylation of the amino-terminal domain in NF-L from OA-treated cultures was seen in both the Triton-soluble and -insoluble subunits, the Triton-soluble subunits were phosphorylated to a higher degree than their insoluble counterparts. The increase ranged from 2-fold for NF-L to 3-fold for NF-M (data not shown).

Since inhibition of PP-2A had already been implicated in the fragmentation of NFs (cf. Fig.1A,B), we examined whether PP-2A$_c$ was capable of acting on NF-M and NF-L from OA-treated cultures. ^{32}P-labelled NF-L and NF-M were immunoprecipitated from control and OA-treated DRG cultures. Prior to their elution from the protein A-sepharose beads the subunits were treated either with purified PP-2A$_c$ or purified PP-1$_c$ in the absence or presence of OA as indicated above each lane. Equal amounts of protein were fractionated by SDS-PAGE. The autoradiograph in Fig.3A shows that PP-2A$_c$ caused a reduction of about 50% in the amount of ^{32}P in both NF-M and NF-L relative to non-phosphatase treated subunits from OA-treated cultures, while OA, at 10 nM, inhibited this PP-2A$_c$-dependent decrease (Fig.3B, compare lanes 3 and 4). Purified PP-1$_c$ had no effect on the ^{32}P content of either subunit (compare lanes 2 and 5).

Since PP-2A had been implicated in the maintenance of NF integrity (Fig.1), we wished to determine whether PP-2A$_c$ could remove the phosphate

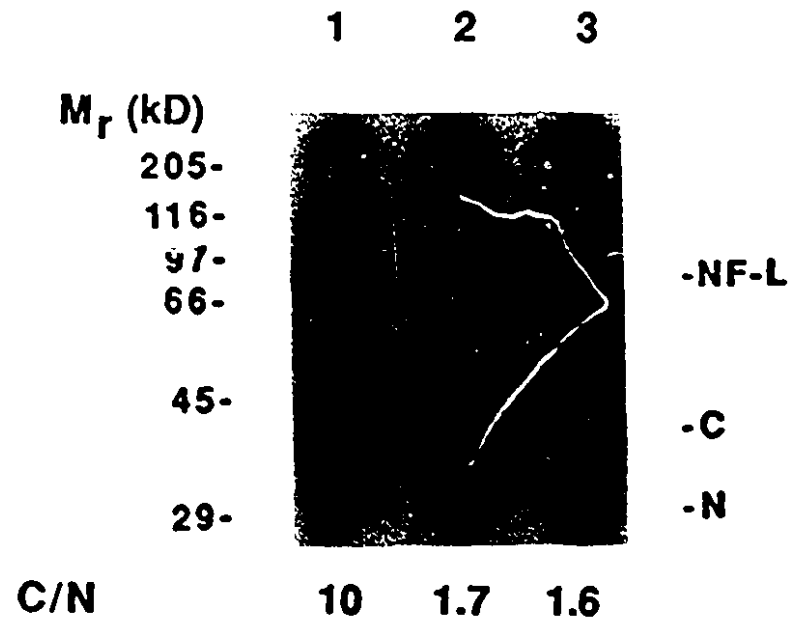
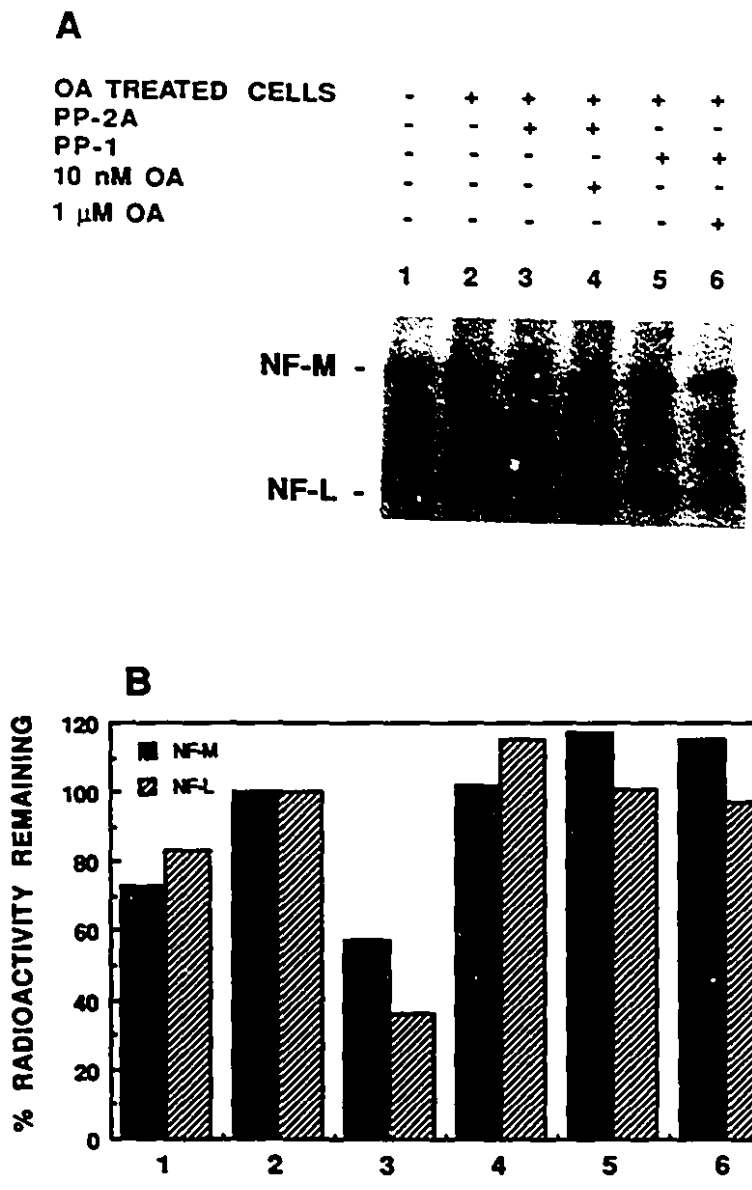


Figure 2: **Gel electrophoretic analysis of the amino- and carboxy-terminal halves of chemically cleaved NF-L from untreated and okadaic acid treated DRG cultures.** DRG cultures were labelled with $^{32}P_i$ for 3 hrs. and either untreated (lane 1) or treated with 1μM OA for 1 hr. (lanes 2 and 3). NF-L was immunoprecipitated from the Triton-insoluble (lane 2) or Triton-soluble (lane 3) fractions. Protein was resolved by SDS-PAGE and digested with NCS (see *Materials and Methods*). *C* and *N* refer to the carboxy-terminal and amino-terminal portions, respectively; of NF-L after NCS treatment. *NF-L* refers to the migration of uncleaved NF-L subunit and the M_r ($x10^{-3}$) of molecular weight standards are shown to the left of the autoradiograph. C/N ratios are shown at the bottom of each lane.

Figure 3: Gel electrophoretic analysis of NF subunits from OA treated DRG cultures after *in vitro* treatment with PP-1_c and PP-$2A_c$. ^{32}P-Labelled NF-L and NF-M were immunoprecipitated from control or OA-treated (1 hr.) DRG cultures (OA). Protein phosphatase treatment (PP-1_c and PP-$2A_c$) was carried out prior to elution of the subunits from the protein A-sepharose beads as described in *Materials and Methods*. Addition of OA during phosphatase treatment at either 10nM or 1μM is indicated above each lane. Equal amounts of the proteins were fractionated by SDS-PAGE and the positions of the 2 subunits are shown to the left of the autoradiograph (panel A). Panel B shows a histogram obtained by quantifying the autoradiograph from panel A. Lanes 1-6 in B correspond to the 6 lanes of the autoradiograph in A. Data pertaining to NF-L are indicated by stippled bars while those for NF-M are represented by solid black bars.


A
OA TREATED CELLS - + + + + +
PP-2A - - + + - -
PP-1 - - - - + +
10 nM OA - - - + - -
1 μM OA - - - - - +
1 2 3 4 5 6
NF-M -
NF-L -
B
% RADIOACTIVITY REMAINING
120
100
80
60
40
20
0
NF-M
NF-L
1 2 3 4 5 6

moieties seen in the amino-terminal domain of NF-L after OA treatment. NF-L bands from the gel in Fig.3A were excised, subjected to NCS digestion and the C/N ratios were determined (Fig.4). Again it can be seen that OA treatment led to an increase in amino-terminal phosphorylation of NF-L, as indicated by the 4.5-fold reduction in the C/N ratio following OA treatment (Fig 4, compare lanes 1 and 2). Treatment of NF-L from OA-treated cultures with PP-$2A_c$ led to an increase in the C/N ratio as compared to untreated subunit, to almost the same level as was seen in NF-L from cultures not treated with OA (Fig.4, compare lanes 1,2 and 3). OA at low concentrations prevented this PP-$2A_c$-dependent reversal of the C/N ratio (Fig.4, lane 4) and PP-1_c had no effect on the ratio indicating that PP-$2A_c$ preferentially removed the OA-induced phosphate moieties from the amino-terminal portion of NF-L.

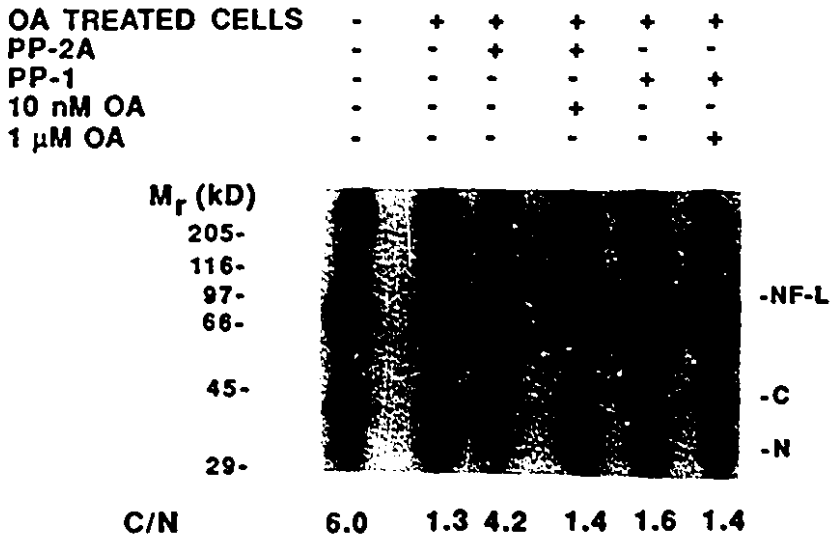


Figure 4: **Gel electrophoretic analysis of chemically cleaved NF-L following *in vitro* treatment with PP-1_c and PP-$2A_c$.** The NF-L subunit from the gel shown in Fig.3 was excised and digested with NCS (see *Materials and Methods*). Treatments are the same as in Fig.3 and are shown above each lane. *C*, *N* and *NF-L* are as indicated in the legend to Fig.2. C/N ratios are shown at the bottom and the M_r ($x10^{-3}$) of molecular weight standards are shown at the left.

Discussion

The fragmentation of NFs that occurs in DRG cultures treated with OA (Sacher *et al*, 1992) may represent the amplification of a mechanism normally involved in modulating the local reorganization of NFs in a variety of situations, including axonal transport. In this respect it is interesting to note that Okabe *et al* (1993) showed a recovery in NF-L fluorescence following photobleaching with no movement of the photobleached zone. Additionally they showed that biotin-labelled NF-L was incorporated into numerous, discrete sites along the axon indicating that soluble oligomers of NF subunits can be transported along the axon faster than the bulk movement of NFs. It is possible that amino-terminal phosphorylation/dephosphorylation allows a transient release of oligomers to occur during the transport of NFs down the axon. The abundance of protein phosphatases in the cell (Hardie *et al*, 1991) would ensure that any local disruption of NFs be of a short duration and fully reversible. Treatment with OA may allow for the accumulation of phosphorylated species which normally have a transient existence.

This model implies that the fragmented NFs should be in a form which is readily re-incorporated into Triton-insoluble structures upon restoration of protein phosphatase activity. Indeed, we have found that the early, reversible stages of OA-induced NF fragmentation give rise to a heterogeneous population of very large oligomers (M.G.S., E.S.A. and W.E.M, in preparation) thus allowing for a rapid recovery of the NF network after OA is removed (Sacher *et al*, 1992).

A major reason for speculating that increased phosphorylation was responsible for the OA-induced fragmentation of NFs was because recent *in vitro* studies showed that IFs composed of vimentin (Geisler *et al*, 1989), GFAP (Inagaki *et al*, 1990), desmin (Geisler and Weber, 1988) or NF-L (Nakamura *et al*, 1990; Gonda *et al*, 1990) were all disrupted upon phosphorylation of the amino-terminal head domain. In addition, second messenger-dependent protein kinases were shown to be involved in phosphorylation of the head domain of NF-L (Sihag and Nixon, 1989) and NF-M (Sihag and Nixon, 1990) leading to the suggestion that head

domain phosphorylation of NFs influences filament assembly states (Nixon and Sihag, 1991).

NCS cleavage of NF-L (Fig.2) showed a correlation between OA treatment and increased amino-terminal domain phosphorylation. Since phosphorylation in the α-helical rod domain of NF subunits has not been reported (Steinert *et al*, 1982), we suspect that the phosphate moieties in the amino-terminal segment of NF-L are located in the head domain.

The OA-induced fragmentation of NFs was attributed to inhibition of PP-2A based on the concentration response data for OA and calyculin A (Fig.1). A similar approach was used to implicate PP-1 in the disruption of the IF network in BHK-21 cells (Eriksson *et al*, 1992). We also showed that PP-$2A_c$, but not PP-1_c, preferentially removed the OA-induced amino-terminal domain phosphorylation sites *in vitro* (Fig.4), further suggesting that inhibition of this enzyme is responsible for the fragmentation of NFs that accompanies OA treatment. However, this result does not exclude the involvement of PP-2A in maintaining the phosphorylation state of the tail domain of NF subunits since the heterotrimeric form of PP-2A was shown to be the most effective form for acting on microtubule-associated protein tau following its phosphorylation by p42 MAP kinase (Goedert *et al*, 1992).

Hisanaga *et al* (1993) showed that PP-2A can dephosphorylate NF-M which had been phosphorylated *in vitro* with protein kinase A. This implies that PP-2A can remove phosphate moieties from the head domain of NF-M since it is this domain which is phosphorylated by protein kinase A *in vitro* (Sihag and Nixon, 1990). Our results showing that PP-$2A_c$ removes phosphate from OA-treated NF-M (Fig.3) indicate that OA treatment also leads to an increase in phosphorylation of the head domain of NF-M, although this remains to be determined directly. We also show a similar dephosphorylation pattern for NF-L. The removal by PP-$2A_c$ of ~50% of the ^{32}P from OA-treated NF-M implies that the enzyme may also dephosphorylate sites in the carboxy-terminal tail domain. The demonstration by Sola *et al* (1991) that PP-2A removes phosphate from Lys-Ser-Pro sequences in histone H1 is consistent with this deduction because similar tripeptide repeats are

found in the tail domain of NF-M (Napolitano *et al*, 1987).

The fact that PP-1_c had no effect on the ^{32}P levels in both NF-L and NF-M is consistent with our studies implicating inhibition of PP-1 in the appearance of electrophoretic variants of all three subunits (Fig.1). These variants were not present in the PP-1_c-treated subunits which were obtained from cells treated with OA for 1 hr. (Fig.4 and Sacher *et al*, 1992). Alternatively, PP-1_c may require additional subunits to be active against NF subunits.

Any attempt to explain the OA-induced fragmentation of NFs in DRG cultures must take into account the similar increase in amino-terminal domain phosphorylation in the Triton-insoluble and soluble subunits (Fig.3), and the higher specific radioactivity of the latter as compared to the former (data not shown). We suggest that hyperphosphorylation of subunits in their amino-terminal domains leads to a relatively slow fragmentation of NFs. The time frame for the disruption of the NF network after OA treatment contrasts with that of the GFAP network, found in the Schwann cells of the DRG cultures, which was shown to be complete within 1 hr. (unpublished observation). The dynamic nature of NFs has been demonstrated by several investigators in recent years (Angelides *et al*, 1989; Okabe *et al*, 1993). These reports have suggested that NFs are in equilibrium with a pool of soluble subunits or oligomers. Phosphorylation of NFs following OA treatment of DRG neurons may lead to a slow accumulation of these soluble oligomers due to amino-terminal domain phosphorylation.

There have been some recent reports suggesting that domain-specific phosphorylation may not be involved in modulating the assembly states of IFs. Chou *et al* (1993) recently showed that there were no differences between the phosphopeptide maps of the soluble and insoluble forms of keratin 8 and keratin 18. One important difference between the soluble keratins in their study and the Triton-soluble NFs in this report is that the keratins were found in a naturally occurring soluble pool made up of tetramers while the OA-induced soluble pool in this study was composed of large oligomers (M.G.S., E.S.A., and W.E.M., in preparation). Nevertheless, these authors proposed a mechanism similar to the one

we invoked above suggesting that phosphorylation may shift the solubility equilibrium constant.

Klymkowsky *et al* (1991) suggested that fragmentation of the cytokeratin network observed during *Xenopus* oocyte maturation may be due to a severing activity similar to the one seen for microtubules (McNally and Vale, 1993). They speculated that phosphorylation may serve to target a severing activity to the filament network giving rise to a heterogeneous population of relatively large oligomers similar to those seen in DRG neurons following OA treatment. In view of the diversity of potential mechanisms that could render IFs Triton-soluble, further studies are required to define the role of domain-specific phosphorylation in NF dynamics.

References

Agostinis,P., Goris,J., Waelkens,E., Pinna,L.A., Marchiori,F., and Merlevede,W. (1987) *J. Biol. Chem.* **262**, 1060-1064

Angelides,K.J., Smith,K.E., and Takeda,M. (1989) *J. Cell Biol.* **108**, 1495-1506

Bialojan,C., and Takai,A. (1988) *Biochem. J.* **256**, 283-290

Caputo,C.B., Sygowski,L.A., Brunner,W.F., Scott,C.W., and Salama,A. (1989) *Biochim. Biophys. Acta* **1012**, 299-307

Chin,S.S., and Liem,R.K.H. (1989) *Eur. J. Cell Biol.* **50**, 475-490

Chin,S.S.M., and Liem,R.K.H. (1990) *J. Neurosci.* **10**, 3714-3726

Chou,C.-F., Riopel,C.L., Rott,L.S., and Omary,B. (1993) *J. Cell Sci.* **105**, 433-444

Chou,Y.-H., Ngai,K.-L., and Goldman,R.D. (1991) *J. Biol. Chem.* **266**, 7325-7328

Cohen,P. (1989) *Annu. Rev. Biochem.* **58**, 453-508

Cohen,P., Alemany,S., Hemmings,B.A., Resink,T.J., Strålfors,P., and Tung,H.Y.L. (1988) *Meth. Enzymol.* **159**, 390-408

Cooper, J.A, Sefton, B.M., and Hunter,T. (1983) *Meth. Enzymol.* **99**, 387-401

Dosemeci,A., Floyd,C.C., and Pant,H.C. (1990) *Cell. Mol. Neurobiol.* **10**, 369-382

Eriksson,J.E., Brautigan,D.L., Vallee,R., Olmsted,J., Fujiki,H., and Goldman,R.D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11093-11097

Geisler,N., Hatzfeld,M., and Weber,K. (1989) *Eur. J. Biochem.* **183**, 441-447

Geisler,N., and Weber,K. (1988) *EMBO J.* **7**, 15-20

Georges,E., Lindenbaum,M.L., Sacher,M.G., Trifaró,J.-M., and Mushynski,W.E. *J. Neurochem.* **52**, 1156-1161

Georges,E., and Mushynski,W.E. (1987) *Eur. J. Biochem.* **165**, 281-287

Goedert,M., Cohen,E.S., Jakes,R., and Cohen,P. (1992) *FEBS Lett.* **312**, 95-99

Gonda,Y., Nishizawa,K., Ando,S., Kitamura,S., Minoura,Y., Nishi,Y., and Inagaki,M. (1990) *Biochem. Biophys. Res. Comm.* **167**, 1316-1325

Grant,N.J., and Aunis,D. (1990) *Eur. J. Cell Biol.* **52**, 36-46

Guan,R.J., Khatra,B.S., and Cohlberg,J.A. (1991) *J. Biol. Chem.* **266**, 8262-8267

Hardie,D.G., Haystead,T.A.J., and Sim,A.T.R. (1991) *Meth. Enzymol.* **201**, 469-476

Hellmich,M.R., Pant,H.C., Wada,E., and Battey,J.F. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 10867-10871

Hisanaga,S., Kusubata,M., Okumura,E., and Kishimoto,T. (1991) *J. Biol. Chem.* **266**, 21798-21803

Hisanaga,S., Yasugawa,S., Yamakawa,T., Miyamoto,E., Ikebe, M., Uchiyama,M., and Kishimoto,T. (1993) *J. Biochem.* **113**, 705-709

Hoffman,P.N., and Lasek,R.J. (1975) *J. Cell Biol.* **66**, 351-366

Inagaki,M., Gonda,Y., Nishizawa,K., Kitamura,S., Sato,C., Ando,S., Tanabe,K., Kikuchi,K., Tsuiki,S., and Nishi,Y. (1990) *J. Biol. Chem.* **265**, 4722-4729

Ingebritsen,T.S., and Cohen,P. (1983) *Eur. J. Biochem.* **132**, 255-261

Ishihara, H., Martin,B.L., Brautigan,D.L., Karaki,H., Ozaki,H., Kato,Y., Fusetani,N., Watabe,S., Hashimoto,K., Uemura,D., and Hartshorne,D.J. (1989) *Biochem. Biophys. Res. Comm.* **159**, 871-877

Julien,J.-P., and Mushynski,W.E. (1982) *J. Biol. Chem.* **257**, 10467-10470

Julien,J.-P., Smoluk,G.D., and Mushynski,W.E. (1983) *Biochim. Biophys. Acta* **755**, 25-31

Klymkowsky,M.W., Maynell,L.A., and Nislow,C. (1991) *J. Cell Biol.* **114**, 787-797

Laemmli,U.K. (1970) *Nature* **227**, 680-685

Lew,J., Winkfein,R.J., Paudel,H.K., and Wang,J.H. (1992) *J. Biol. Chem.* **267**, 25922-25926

Lindenbaum,M.L., Carbonetto,S., and Mushynski,W.E. (1987) *J. Biol. Chem.* **262**, 605-610

Lischwe,M.A., and Ochs,D. (1982) *Anal. Biochem.* **127**, 453-457

Mahboub,S., Richard,C., Delacourte,A., and Han,K.-K. (1986) *Anal. Biochem.* **154**, 171-182

Matsuoka,Y., Nishizawa,K., Yano,T., Shibata,M., Ando,S., Takahashi,T., and

Inagaki,M. (1992) *EMBO J.* **11**, 2895-2902

McNally,F.J., and Vale,R.D. (1993) *Cell* **75**, 419-429

Nakamura,Y., Takeda,M., Angelides,K.J., Tanaka,T., Tada,K., and Nishimura,T. (1990) *Biochem. Biophys. Res. Comm.* **169**, 744-750

Napolitano,E.W., Chin,S.S.M., Colman,D.R., and Liem,R.K.H. (1987) *J. Neurosci.* **7**, 2590-2599

Nixon,R.A., and Sihag,R.K. (1991) *Trends Neurosci.* **14**, 501-506

Okabe,S., Miyasaka,H., and Hirokawa,N. (1993) *J. Cell Biol.* **121**, 375-386

Sacher,M.G., Athlan,E.S., and Mushynski,W.E. (1992) *Biochem. Biophys. Res. Comm.* **186**, 524-530

Shea,T.B., Paskevich,P.A., and Beermann,M.L. (1993) *J. Neurosci. Res.* **35**, 507-521

Shetty,K.T., Link,W.T., and Pant,H.C. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6844-6848

Shetty,K.T., Veeranna, and Guru,S.C. (1992) *Neurosci. Lett.* **137**, 83-86

Sihag,R.K., and Nixon,R.A. (1989) *J. Biol. Chem.* **264**, 457-464

Sihag,R.K., and Nixon,R.A. (1990) *J. Biol. Chem.* **265**, 4166-4171

Sihag,R.K., Jeng,A.Y., and Nixon,R.A. (1988) *FEBS Lett.* **233**, 181-185

Sola,M.M., Langan,T., and Cohen,P. (1991) *Biochim. Biophys. Acta* **1094**, 211-216

Steinert,P.M., and Roop,D.R. (1988) *Annu. Rev. Biochem.* **57**, 593-625

Steinert,P.M., Wantz,M.L., and Idler,W.W. (1982) *Biochemistry* **21**, 177-183

Swanstrom,R., and Shank,P.R. (1978) *Anal. Biochem.* **86**, 184-192

Toru-Delbauffe,D., and Pierre,M. (1983) *FEBS Lett.* **162**, 230-234

Vallano,M.L., Buckholz,T.M., and DeLorenzo,R.J. (1985) *Biochem. Biophys. Res. Comm.* **130**, 957-963

Wible,B.A., Smith,K.E., and Angelides,K.J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 720-724

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Chapter 4

CHARACTERIZATION OF FRAGMENTED NEUROFILAMENTS FORMED IN NEURONS TREATED WITH OKADAIC ACID

Summary

Treatment of rat dorsal root ganglion cultures with the protein phosphatase inhibitor, okadaic acid (OA), leads to a fragmentation of neurofilaments (NFs) as well as a reduction in the mobilities of all three NF subunits on SDS-polyacrylamide gels (Sacher,M.G., Athlan,E.S. and Mushynski,W.E. (1994) *J. Biol. Chem.* **269**, 14,480-14,484). Treatment of the slower migrating form of the low molecular weight subunit (NF-L) with the catalytic subunit of either protein phosphatase-1 or protein phosphatase-2A restored its mobility to that of native NF-L indicating that phosphate moieties regulated by both enzymes may be involved in the reduction in electrophoretic mobility. In addition, OA treatment led to an increase in amino-terminal domain phosphorylation of the mid-sized subunit (NF-M) similar to that previously observed for NF-L. Immunoprecipitation under non-denaturing conditions of Triton X-100-soluble NF fragments from OA-treated neurons demonstrated the existence of oligomers consisting of NF-L and NF-M or NF-L and the high molecular weight subunit (NF-H). A similar immunoprecipitation pattern was observed with the small, naturally occurring Triton X-100-soluble pool of NFs in untreated DRG neurons. These results indicate that intermediates involved in NF assembly may be heterooligomers composed of NF-L associated with either NF-H or NF-M.

Introduction

The intermediate filament (IF) composition of the neuronal cytoskeleton changes during development (Nixon and Shea, 1992). Neurofilaments (NFs), the IF class expressed in mature neurons, are composed of three phosphoprotein subunits referred to as NF-L (62,000 Da), NF-M (95,000 Da), and NF-H (115,000 Da). NF-M and NF-H are two of the most highly phosphorylated proteins known, with the rat subunits containing 8 and 14 moles of phosphate per polypeptide, respectively (Julien and Mushynski, 1982). As members of the IF family of proteins, NF subunits contain several identifiable structural features. In addition to the amino-terminal "head" domain and carboxy-terminal "tail" domain, all IF proteins contain a central "rod" domain rich in α-helical content (see Steinert and Roop, 1988, Weber and Fuchs, 1994, for review). While most of the phosphate moieties on NF-M and NF-H reside in the tail domain (Julien and Mushynski, 1983) low levels of phosphate have been detected outside of this region as well (Pleasure *et al*, 1990; Sihag and Nixon, 1990).

The initial stage of IF assembly involves the formation of a coiled-coil dimer consisting of two identical (eg. vimentin) or non-identical (eg. basic/acidic keratins) subunits. These dimers then go on to form tetramers, octamers and eventually a 10 nm wide filament (see Weber and Fuchs, 1994, and Heins and Aebi, 1994, for review). A cross-section of an IF is thought to contain as many as 32-41 subunits (Albers and Fuchs, 1992; Heins *et al*, 1993).

All three NF subunits have been shown to be capable of assembling into homopolymeric filaments *in vitro* (Tokutake *et al*, 1984; Balin and Lee, 1991). The "tail" domains of NF-M and NF-H project laterally from the filament axis and appear to contact adjacent filaments (Hisanaga and Hirokawa, 1988; Gotow *et al*, 1992). NF-L is thought to form the core of NFs as deduced from immunoelectron microscopic data (Sharp *et al*, 1982) and the observation that it is the only subunit capable of forming long, 10 nm-wide filaments *in vitro* (Geisler and Weber, 1981; Tokutake *et al*, 1984; Balin and Lee, 1991). In addition, NF-M and NF-H were

unable to co-assemble in an IF-deficient (IF$^-$) cell line (Ching and Liem, 1993) or in the absence of assembly competent NF-L in neurons of a quail mutant known as "quiver" (Ohara *et al*, 1993). NFs were recently shown to be obligate heteropolymers *in vivo* (Lee *et al*, 1993) and substoichiometric amounts of NF-M were sufficient to drive the formation of a NF network when co-expressed with NF-L in IF$^-$ cells. Although these studies have elucidated some important general rules governing NF assembly there is still much to be learned about the actual mechanism of assembly and the distribution of the individual subunits within NFs.

Recently we showed that the treatment of cultured rat dorsal root ganglion (DRG) neurons with the protein phosphatase-1 and -2A (PP-1 and PP-2A, respectively) inhibitor, okadaic acid (OA), led to a fragmentation of the NF network (Sacher *et al*, 1992,1994) as evidenced by the increased Triton X-100 (Triton) solubility of all three NF subunits. The present study is directed at the further characterization of these Triton-soluble NF fragments. Our results show the presence of heterooligomers of NF-L associated with either NF-M or NF-H. Such heterooligomers were also observed in the small, naturally occurring pool of Triton-soluble NFs suggesting that OA amplifies a naturally occurring process. OA treatment led to the initial release of large NF fragments which were Triton-soluble at 13,000x*g* but insoluble at 100,000x*g*. Finally, the catalytic subunits of both PP-1 and PP-2A were capable of restoring the gel electrophoretic mobilities of slower migrating forms of NF subunits induced by OA treatment (Sacher *et al*, 1992) back to the mobilities of NF subunits from untreated neurons.

Materials and Methods

Materials: $^{32}P_i$-Orthophosphoric acid was from ICN (Mississauga,ON). The catalytic subunits of PP-1 and PP-2A were prepared as described by Cohen *et al* (1988). OA was from LC Services (Woburn,MA).

Cell Culture: Rat DRGs were dissected, dispersed and maintained in defined medium as previously described (Sacher *et al*, 1992). For metabolic labelling cells were incubated with 0.5 mCi of carrier-free $^{32}P_i$ per mL P_i-free medium (Flow Laboratories, McLean, VA) for 3 hrs. prior to OA treatment.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE): Gel electrophoresis and Western blotting were performed as previously described (Laemmli, 1970; Sacher *et al*, 1992). Polypeptides used for analysis by chemical cleavage (see below) were obtained from SDS-polyacrylamide gels run in the presence of 0.025% thioglycolic acid to minimize protein oxidation.

Two-dimensional phosphopeptide mapping: Immunoprecipitated, ^{32}P-labelled NF-M, resolved by SDS-PAGE, was located by autoradiography, excised and the gel slice was washed with 20% methanol. Gel slices were digested in 0.5 mL of 50 mM ammonium bicarbonate with 10 μg TLCK-α-chymotrypsin (Sigma Chemical Co.,St. Louis,MO) for 18 hrs. followed by 10 μg TPCK-Trypsin for 6 hrs. Gel slices were removed and the digest was lyophilized and dissolved in H_2O. Equal ^{32}P-counts were spotted onto 20cm X 20cm cellulose sheets (MN-300, 0.1mm; Brinkman,Westbury,NY). Electrophoresis was performed for 40 min. at 1 kV in water:acetic acid:formic acid (80:15:5). Chromatography in the second dimension was performed in butanol:pyridine:acetic acid:water (75:50:15:60) as described previously (Sihag and Nixon, 1990). Phosphopeptides were visualized by autoradiography using a Dupont Lightning-plus intensifying screen (Swanstrom and Shank, 1978).

Cleavage of NF-M with Nitrothiocyanobenzoic acid (NTCB): Immunoprecipitated, ^{32}P-labelled NF-M was resolved by SDS-PAGE, located by autoradiography and excised. The gel slice was washed extensively with water, equilibrated in 2-nitro-5-thiocyanobenzoic acid (NTCB)-buffer (6M urea, 50 mM TrisHCl pH 8.0), then incubated for 1 hr. at room temperature in NTCB-buffer containing 6 mM NTCB at which time the pH was raised to 9.5 by addition of Tris base. The slices were then incubated at 37°c overnight followed by extensive washing in water and equilibration in SDS-PAGE sample buffer and were then loaded vertically onto an SDS-10%-polyacrylamide gel. Polypeptides were localized and subjected to two-dimensional phosphopeptide mapping as described above.

Immunoprecipitation: Cells were harvested in cytoskeleton extraction buffer (CSK buffer) containing 1% Triton X-100 (Triton), 150 mM NaCl, 50 mM TrisHCl pH 7.5, 50 mM NaF, 2 mM EGTA, 2 mM levamisol, 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitation was carried out as described previously (Sacher *et al*, 1994). The following monoclonal antibodies from Sigma Chemical Co. (St. Louis,MO) were used: NE14 (anti-NF-H), NN18 (anti-NF-M), and NR4 (anti-NF-L). For immunoprecipitating radiolabelled NF-M a rabbit polyclonal antiserum was used. Immunoprecipitation under non-denaturing conditions was carried out essentially as described by Sacher *et al* (1994) except that SDS was not added to the samples.

Phosphatase treatment of immunoprecipitated proteins: DRG cultures were treated with 1 μM OA for 6 hrs. and NFs were immunoprecipitated as described above. Samples were incubated for 5 hrs. at 30°c, prior to elution from the protein A-sepharose beads, in 65 mM TrisHCl pH 7, 1 mM $MgCl_2$, 0.5 mM EGTA, 1 mM PMSF with or without $PP\text{-}2A_c$ (1.5 μg/mL), $PP\text{-}1_c$ (1.5 μg/mL) and OA as described in the figure legend.

Gel Filtration: DRG cultures were treated with 1μM OA for 1 hr. and harvested

in CSK buffer as described above. 300 μL samples were loaded onto a 5.5 mL Biogel A50M (BioRad,Hercules,CA) column (8.5 cm X 0.9 cm), fractions of 250 μL were collected and NF subunits were detected by Western blotting as described above.

Results

Previous studies in our laboratory have shown an increase in Triton-soluble NFs following treatment with 1μM OA. To begin characterizing these soluble species we examined their relative sizes by differential centrifugation. DRG cultures were treated with 1μM OA for increasing times, harvested in buffer containing 1% Triton, and centrifuged at either 13,000x*g* or 100,000x*g*. The Triton-insoluble pellets and Triton-soluble supernatants were then examined for the presence of the three NF subunits (Fig.1). At 13,000x*g* most of the subunits were found in the Triton-soluble fraction within 3 hrs. of OA treatment. The NF fragments that remained in the supernatant following centrifugation of Triton extracts at 13,000x*g* were almost completely pelleted at 100,000x*g*. The presence of a significant proportion of NF proteins in the supernatant fraction obtained after centrifuging Triton extracts at 100,000x*g* was seen only after 6 hrs. of OA treatment. It is interesting to note that the slower migrating form of NF-L seen after 6 hrs. of OA treatment was found only in the Triton-soluble fraction at both 13,000x*g* and 100,000x*g*.

To assess whether NFs were being fragmented in a random fashion or broken into fragments of a more defined size DRG cultures were treated with 1μM OA for 30 min. and 60 min. and the Triton-soluble extracts were fractionated on a BioGel A50M gel filtration column having a size exclusion limit of $50x10^6$ daltons. In both cases all three NF subunits were found distributed over a wide range of fractions indicating that the oligomers were heterogeneous in size and implying a random fragmentation of NFs during OA treatment (data not shown).

We have previously shown an increase in phosphorylation of the amino-terminal domain of NF-L from DRG cultures exposed to 1μM OA (Sacher *et al*, 1994). To determine whether OA treatment caused the appearance of new phosphorylation sites in NF-M duplicate DRG cultures were pre-labelled with $^{32}P_i$ for 3 hrs. and incubated in the presence or absence of 1μM OA for 1 hr. Fig. 2 shows the two-dimensional phosphopeptide maps of NF-M from untreated (Fig.2A), OA treated, Triton-insoluble (Fig.2B) and OA treated, Triton-soluble

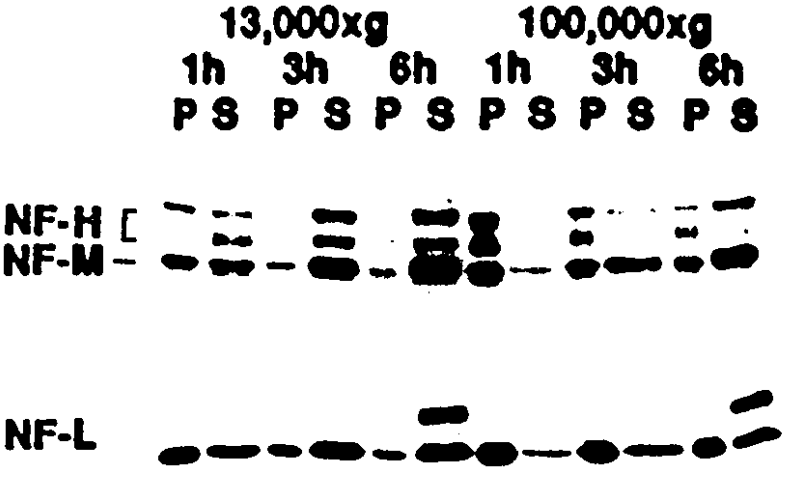
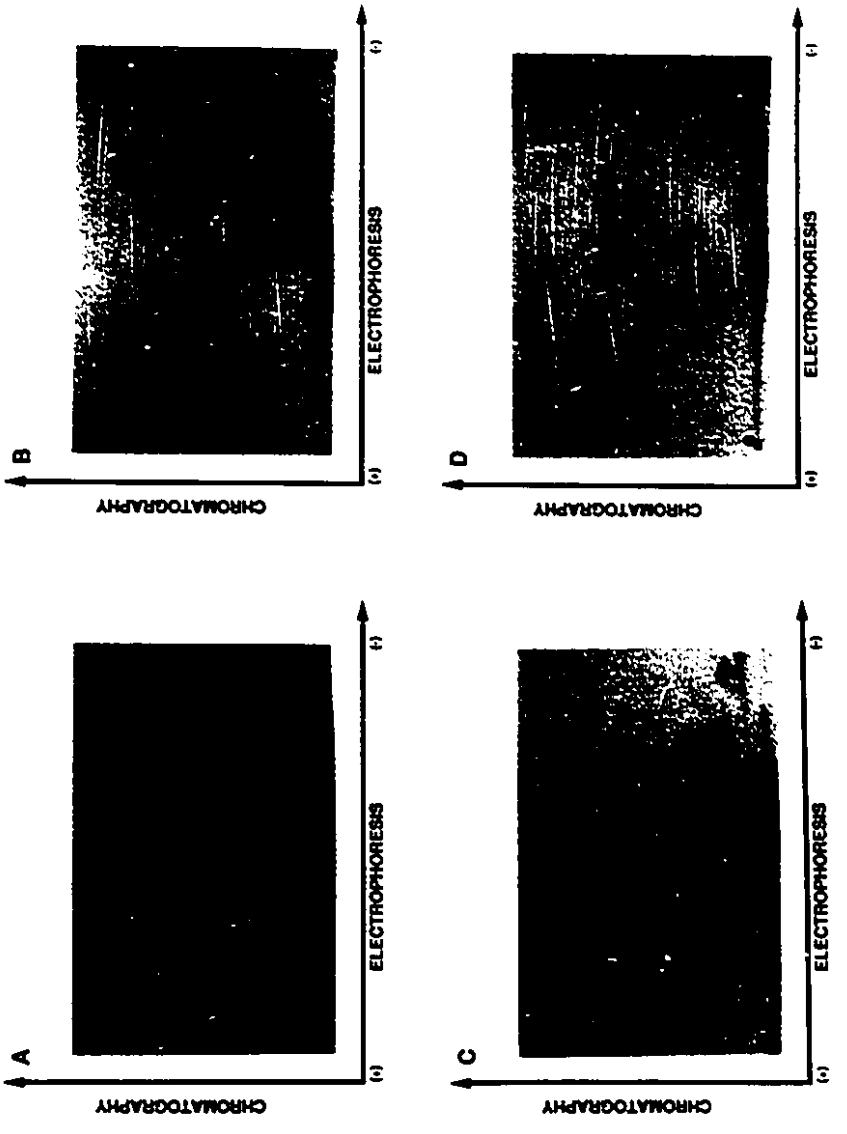


Figure 1: Differential solubility of NFs from OA-treated neurons. DRG cultures were treated with 1μM OA for the times indicated, harvested in CSK buffer and centrifuged at either 13,000x*g* or 100,000x*g*. Pellets (P) and supernatants (S) were fractionated by SDS-PAGE and detected by Western blotting. *NF-L*, *pNF-L*, *NF-M*, and *NF-H* refer to the low, hyperphosphorylated low, middle, and high molecular weight NF subunits, respectively.

Figure 2: Two-dimensional phosphopeptide mapping of NF-M. DRG cultures were pre-labelled with $^{32}P_i$ for 3 hrs. and were either left untreated (A) or were treated with 1μM OA for 1 hr. (B-D). NF-M was immunoprecipitated, resolved by SDS-PAGE and subjected to two-dimensional phosphopeptide mapping as described in *Materials and Methods*. Panels B and C represent NF-M from Triton-insoluble and Triton-soluble fractions, respectively. Panel D represents the phosphopeptide map of the carboxy-terminal portion of NF-M obtained by NTCB cleavage. The origin is indicated by *O* and the arrowheads point to the 4 prominent phosphopeptides seen in the full length subunit after OA treatment. (E) The location of the two cysteine residues in NF-M as well as the expected fragments are shown. The curved line indicates the amino-terminal head domain, the stippled box represents the α-helical rod domain and the open box represents the carboxy-terminal tail domain.


B
CHROMATOGRAPHY
ELECTROPHORESIS
(+)
(-)
D
CHROMATOGRAPHY
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A
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ELECTROPHORESIS
(+)
(-)
C
CHROMATOGRAPHY
ELECTROPHORESIS
(+)
(-)

E

FRAGMENT	SIZE (kDa)
$NH_2 \rightarrow C_{278}$	31.4
$NH_2 \rightarrow C_{293}$	33.2
$C_{278} \rightarrow COOH$	64.3
$C_{293} \rightarrow COOH$	62.4
$C_{278} \rightarrow C_{293}$	1.9

(Fig.2C) fractions. Four species, designated as phosphopeptides 1, 2, 3 and 4, were seen in the peptide maps of NF-M from both the Triton-insoluble and -soluble fractions of OA-treated cultures. Although phosphopeptides 1 and 2 seem to appear faintly in the untreated sample (Fig.2A) they are more apparent after cultures were treated with OA. The location of these four phosphopeptides in NF-M was determined using ^{32}P-labelled NF-M immunoprecipitated from OA-treated DRG cultures and subjected to NTCB digestion (Fig.2E). Two-dimensional phosphopeptide mapping of the carboxy-terminal fragments (Fig.2D), which extend from either cysteine-278 or cysteine-293 to the carboxy-terminus, showed that they lacked all 4 phosphopeptides seen in the phosphopeptide maps of full length NF-M from OA-treated cultures (compare Fig.2D with Fig.2B and Fig.2C). These results indicate that the latter are located in the amino-terminal domain of NF-M. The amino-terminal fragments, extending from the amino-terminus to either cysteine-278 or cysteine-293 (Fig.2E), were too weakly labelled following NTCB digestion and could not be used for two-dimensional phosphopeptide mapping.

Treatment of DRG cultures with 1μM OA for a minimum of 3 hrs. leads to the appearance of slower migrating forms of all three NF subunits (Fig.1; Sacher *et al*, 1992,1994). We previously inferred from the response to varying concentrations of OA that inhibition of PP-1 activity caused the appearance of these slower migrating forms. To directly test this notion DRG cultures were treated with 1μM OA for 6 hrs. and both NF-L and NF-M were immunoprecipitated. The subunits were dephosphorylated with either the catalytic subunit of PP-1 (PP-1$_c$) or PP-2A (PP-2A$_c$) (Fig.3). Surprisingly, either PP-2A$_c$ or PP-1$_c$ caused the disappearance of the slower migrating form of NF-L (pNF-L) and sharpening of the broad band formed by NF-M (Fig.3, compare lane 1 with lanes 2 and 4).

The OA-induced fragmentation of NFs allowed us to examine the organization of the NF network, in DRG neurons, by analyzing subunit interactions under non-denaturing conditions. To this end DRG cultures were treated with 1μM OA for 1 hr., harvested in CSK buffer, and the Triton-soluble NFs were either

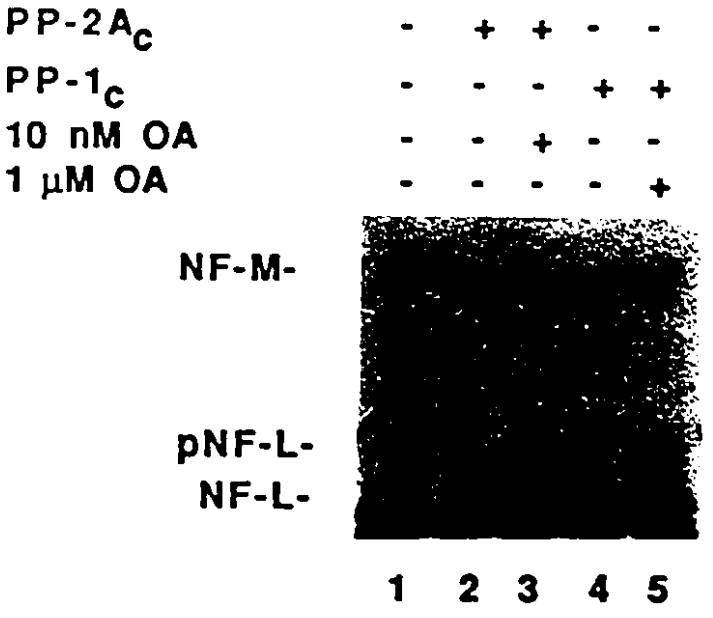

Figure 3: Treatment of hyperphosphorylated NFs with PP-1_c and PP-$2A_c$. DRG cultures were treated with OA for 6 hrs. at which time NF-L and NF-M were immunoprecipitated. Prior to elution of the subunits from the protein A-sepharose beads samples were untreated (lane 1) or treated with either PP-1_c (lanes 4 and 5) or PP-$2A_c$ (lanes 2 and 3) with (lanes 3 and 5) or without (lanes 1, 2 and 4) OA as indicated above each lane. *NF-L*, *pNF-L*, and *NF-M* are as indicated in the legend to figure 1.

boiled in the presence of SDS or left untreated. Samples were then immunoprecipitated with one of three monoclonal antibodies (see *Materials and Methods*) which immunoprecipitate specific NF subunits (data not shown). In the presence of SDS each antibody immunoprecipitated its specific subunit (Fig.4, lanes L/+, M/+ and H/+). In addition the anti-NF-M and anti-NF-H antibodies immunoprecipitated small amounts of NF-L (Fig.4, lanes M/+ and H/+), perhaps due to the partial removal of SDS by Triton micelles and the subsequent reassociation of some of the subunits. In the absence of SDS the anti-NF-M and anti-NF-H antibodies each immunoprecipitated much larger amounts of NF-L in addition to the corresponding subunit (Fig.4, lanes M/- and H/-). The anti-NF-L antibody immunoprecipitated both NF-M and NF-H (Fig.4, lane L/-) yielding a pattern similar to that of the anti-NF-M and anti-NF-H lanes combined. These results indicate the existence of units consisting of NF-L associated predominantly with either NF-M or NF-H.

We then wished to determine whether the NF subunit associations seen after 1 hr. OA treatment reflected the early fragmentation pattern as well as the later pattern. Immunoprecipitation under denaturing and non-denaturing conditions was carried out, following 1μM OA treatment for increasing times, with 13,000x*g* Triton-soluble NFs. The association of NF-L with NF-M or NF-H was seen for all time points examined (data not shown) implying that these heterooligomers may constitute intermediates in NF assembly and disassembly in rat DRG neurons. If this is true then we would expect to see a similar immunoprecipitation pattern using NFs in a much smaller oligomeric state. DRG cultures treated with 1μM OA for 6 hrs. give rise to a large pool of 100,000x*g* Triton-soluble subunits (cf. Fig.1). These small, oligomeric units were then subjected to immunoprecipitation as described above. Once again the pattern seen under non-denaturing conditions was indistinguishable from that obtained using 13,000x*g* Triton-soluble NFs (Fig.5) implying that these small oligomers were composed of heteromers of NF-L with either NF-M or NF-H.

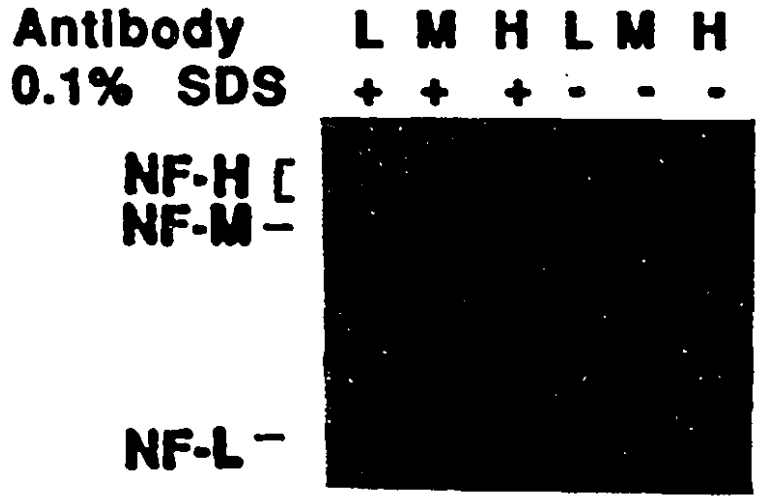

Figure 4: Immunoprecipitation of OA-induced, 13,000x*g* Triton soluble NFs. DRG cultures were treated with 1μM OA for 1 hr. and harvested in CSK buffer. Supernatants treated with SDS (indicated above each lane) were boiled in the presence of 0.3% SDS for 2 min. prior to immunoprecipitation. *NF-L*, *NF-M*, and *NF-H* refer to the migration of the three NF subunits. *H*, *M*, and *L* above each lane indicate the NF subunit antibody used for immunoprecipitation (see *Materials and Methods*).

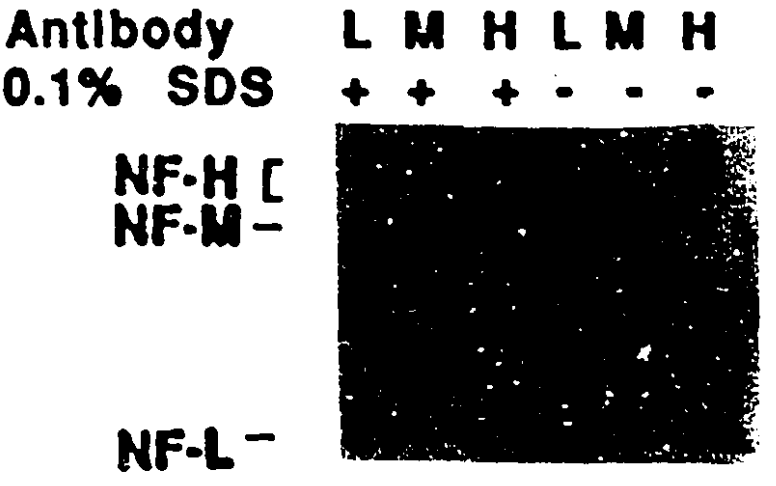

Figure 5: Immunoprecipitation of OA-induced, 100,000xg Triton soluble NFs. DRG cultures were treated with 1μM OA for 6 hrs., harvested in CSK buffer at 100,000x*g* and immunoprecipitated in the presence or absence of SDS (indicated above each lane) as described in *Materials and Methods*. *NF-L*, *NF-M*, *NF-H*, *H*, *M*, and *L* are as described in the legend to figure 4.

Previously (Sacher *et al*, 1994) we speculated that the OA-induced fragmentation of NFs may represent the amplification of a mechanism for localized NF disassembly. A small pool of 13,000*xg* Triton-soluble NFs is present in untreated DRG cultures (Sacher *et al*, 1992, 1994). Using the immunoprecipitaion procedure described above we examined whether the same NF subunit associations existed in these untreated oligomers. Under non-denaturing conditions we noticed, as with OA treated Triton-soluble NFs, an association between NF-L and the larger subunits, although some NF-H co-immunoprecipitated with NF-M and NF-L when using anti-NF-M antibody and some NF-M co-immunoprecipitated with the two other subunits when using anti-NF-H antibody (Fig.6). Note, however, that the ratio of NF-H to NF-M was higher in the immunoprecipitate obtained with anti-NF-H antibody (Fig.6).

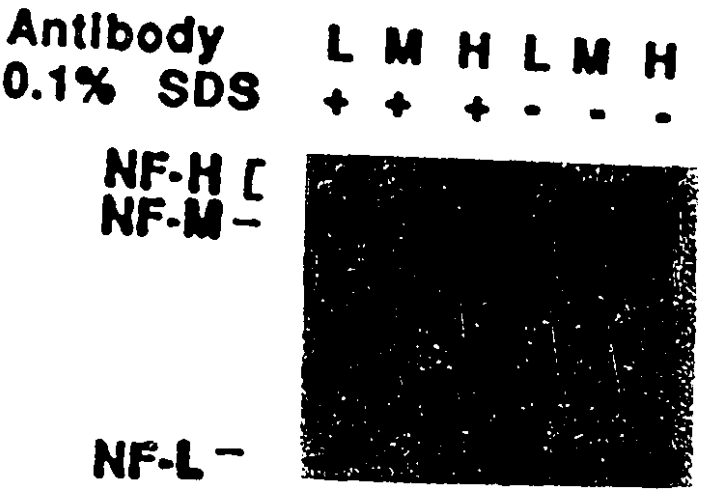

Figure 6: Immunoprecipitation of Triton soluble NFs from untreated neurons. DRG cultures were harvested in CSK buffer at 13,000x*g* and immunoprecipitated in the presence or absence of SDS (indicated above each lane) as described in *Materials and Methods*. *NF-L*, *NF-M*, *NF-H*, *H*, *M*, and *L* are as described in the legend to figure 4.

Discussion

Treatment of DRGs with OA caused an increase in the phosphorylation of the amino-terminal domain of NF-M (Fig.2) similar to that seen in NF-L (Sacher *et al*, 1994) as well as a fragmentation of NFs (Sacher *et al*, 1992). The appearance of two faint phosphopeptides in the sample obtained from untreated cultures (Fig.2A) probably reflects the low amount of phosphate previously reported in this region of the protein (Sihag and Nixon, 1990). It is apparent, however, that OA treatment causes an increase in amino-terminal head domain phosphorylation. Since NF-L appears to be the subunit responsible for allowing NF formation to proceed (Ching and Liem, 1993; Lee *et al*, 1993; Ohara *et al*, 1993) the significance of head domain phosphorylation in NF-M is unclear.

A fragmentation of native NFs similar to that seen in OA-treated DRGs was recently reported following *in vitro* phosphorylation by cAMP-dependent protein kinase (Hisanaga *et al*, 1994). In this case as well NF-L was shown to be phosphorylated in the amino-terminal head domain. The same study showed that NFs reassembled *in vitro* were broken into smaller oligomers than were native NFs indicating that other proteins may be involved in maintaining NFs in an assembled state or in mediating NF assembly *in vivo*.

The ability of both PP-$2A_c$ and PP-1_c to cause the disappearance of the slower migrating forms of NF-L (pNF-L) and NF-M (Fig.3) may be due to a removal of the same phosphate moieties, as the two enzymes have been shown to have a broad and overlapping range of substrate specificities (Cohen, 1989). For example, a brain-specific inhibitor of PP-1 (DARPP-32) that has been phosphorylated by casein kinase-2 is a substrate for both PP-1 and PP-2A (Girault *et al*, 1989). It is not clear whether the same phosphate moieties are removed from this protein by the two phosphatases, although Agostinis *et al* (1987) showed that both PP-2A and PP-1 were capable of dephosphorylating the same mono-phosphorylated peptide, albeit at different rates. An alternative explanation for these results is that several phosphate moieties, some regulated by PP-1 others by

PP-2A, may be involved in reducing the mobilities of the NF subunits and that removal of either type of phosphate causes a return to the mobilities seen for native subunits. This could be verified by selectively inhibiting PP-1 *in vivo* using the heat stable inhibitors I1 and I2 (Aitken *et al*, 1982; Holmes *et al*, 1986) and looking for the appearance of slower migrating NF subunits. It is interesting to note that chronic treatment of rat DRGs with 10-20 nM OA does not cause the appearance of slower migrating forms of NF subunits yet the amino-terminal phosphorylation patterns for both NF-L and NF-M are identical to those of subunits from cultures treated with 1 μM OA (M.G.S., unpublished observations). Since low OA concentrations inhibit PP-2A more effectively than PP-1, this result implies that inhibition of PP-2A alone is not sufficient to cause a reduction in NF subunit mobilities.

It has long been known that NF subunits can co-assemble with other IF proteins in transfected cells (Chin and Liem, 1989,1990; Gill *et al*, 1990; Wong and Cleveland, 1990; Soifer *et al*, 1991; Nash and Carden, 1991). However, in IF cells neither of the three NF subunits could form homopolymers (Ching and Liem, 1993; Lee *et al*, 1993) suggesting that NFs are obligate heteropolymers *in vivo*. The co-immunoprecipitation studies show an association between NF-L and either NF-M or NF-H (Fig.4). Our failure to observe individual subunits after immunoprecipitation under non-denaturing conditions, even when working with oligomers soluble in Triton at 100,000x*g*, implies that these heterooligomers may represent intermediates involved in the assembly and disassembly of NFs. These data may explain the results of Ching and Liem (1993) and Lee *et al* (1993): namely, that NF formation cannot proceed in the presence of NF-L alone. Moreover, the importance of NF-L in driving NF formation is demonstrated by the inability of NF-M and NF-H to co-assemble *in vivo* (Ching and Liem, 1993; Ohara *et al*, 1993). Consistent with this finding, we were able to detect increased levels of associated NF-L following immunoprecipitation under non-denaturing conditions using either anti-NF-M or anti-NF-H antibodies (Figs.4-6).

Heterooligomers of NF-L and NF-M or NF-L and NF-H have never previously been reported *in vivo*. Carden and Eagles (1986) did report the presence of heterodimers of NF-L and NF-M following disassembly of NFs at low pH and low ionic strength conditions. Mulligan *et al* (1991) suggested that heterooligomers consisting of NF-L with either of the two larger NF subunits could explain all reassembly and immunoelectron microscopic data. Our co-immunoprecipitation studies now provide biochemical evidence for the existence of such heteromers *in vivo*.

Untreated DRG cultures contain a small pool of NF oligomers soluble in Triton at 13,000x*g*. Immunoprecipitation under non-denaturing conditions using an anti-NF-M antibody revealed the presence of oligomers containing NF-H and NF-L in addition to NF-M (Fig.6). However, the ratio of NF-M to NF-H changed when an anti-NF-H antibody was used instead (Fig.6). The presence of NF-H in addition to NF-L and NF-M in the sample from untreated cultures immunoprecipitated with an anti-NF-M antibody contrasts with its absence in OA treated DRGs (Figs.4 and 5). Perhaps the discrepancy is simply due to a difference in the size range of the NF fragments in the two populations. In addition repulsive forces due to charged amino acid residues and phosphorylation of the tail domains of NF-M and NF-H may be further increased by hyperphosphorylation of the Triton-soluble NF fragments from OA treated cultures rendering them more fragile and increasing the likelihood that the peripherally associated NF-H subunit will dissociate. The increased ratio of NF-H to NF-M seen using the anti-NF-H antibody (Fig.6) implies that, while some of the NF-H subunit is associated with NF-L/NF-M heterooligomers, NF-H/NF-L heterooligomers are also present.

The present results provide the first *in vivo* biochemical evidence for the existence of heterooligomers consisting of NF-L associated predominantly with either NF-M or NF-H. Clearly, this tissue culture system can be further exploited to clarify the unresolved question of a cause and effect relationship between head domain phosphorylation of NF subunits and NF assembly.

References

Agostinis,P., Goris,J., Waelkens,E., Pinna,L.A., Marchiori,F. and Merlevede,W. (1987) *J. Biol. Chem.* **262**, 1060-1064

Aitken,A., Bilham,T. and Cohen,P. (1982) *Eur. J. Biochem.* **126**, 235-246

Albers,K. and Fuchs,E. (1992) *Int. Rev. Cytol.* **134**, 243-279

Balin,B.J. and Lee,V.M.-Y. (1991) *Brain Res.* **556**, 196-208

Carden,M.J. and Eagles,P.A.M. (1986) *Biochem. J.* **234**, 587-591

Ching, G.Y. and Liem,R.K.H. (1993) *J. Cell Biol.* **122**, 1323-1335

Chin,S.S.M. and Liem,R.K.H. (1990) *J. Neurosci.* **10**, 3714-3726

Chin,S.S.M. and Liem,R.K.H. (1989) *Eur. J. Biochem.* **50**, 475-490

Cohen,P., Alemany,S., Hemmings,B.A., Resink,T.J., Strålfors,P. and Tung,H.Y.L. (1988) *Meth. Enzymol.* **159**, 390-408

Geisler,N. and Weber,K. (1981) *J. Mol. Biol.* **151**, 565-571

Gill,S.R., Wong,P.C., Monteiro,M.J. and Cleveland,D.W. (1990) *J. Cell Biol.* **111**, 2005-2019

Girault,J.A., Hemmings,H.C.Jr., Williams,K.R., Nairn,A.C. and Greengard,P. (1989) *J. Biol. Chem.* **264**, 21,748-21,759

Gotow,T., Takeda,M., Tanaka,T. and Hashimoto,P.H. (1992) *Eur. J. Cell Biol.* **58**, 331-345

Heins,S. and Aebi,U. (1994) *Curr. Opin. Cell Biol.* **6**, 25-33

Heins,S., Wong,P.C., Müller,S., Goldie,K., Cleveland,D.W. and Aebi,U. (1993) *J. Cell Biol.* **123**, 1517-1533

Hisanaga,S., Matsuoka,Y., Nishizawa,K., Saito,T., Inagaki,M. and Hirokawa,N. (1994) *Mol. Biol. Cell* **5**, 161-172

Hisanaga,S. and Hirokawa,N. (1988) *J. Mol. Biol.* **202**, 297-305

Holmes,C.F.B., Campbell,D.G., Caudwell,F.B., Aitken,A. and Cohen,P. (1986) *Eur. J. Biochem.* **155**, 173-182

Julien,J.-P. and Mushynski,W.E. (1982) *J. Biol. Chem.* **257**, 10,467-10,470

Laemmli,U.K. (1970) *Nature* **227**, 680-685

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

Mulligan,L., Balin,B.J., Lee,V.M.-Y. and Ip,W. (1991) *J. Struct. Biol.* **106**, 145-160

Nash,J.A.B. and Carden,M.J. (1991) *Biochem. Soc. Trans.* **19**, 1147-1148

Nixon,R.A. and Shea,T.B. (1992) *Cell Motil. Cytoskel.* **22**, 81-91

Ohara,O., Gahara,Y., Miyake,T., Teraoka,H. and Kitamura,T. (1993) *J. Cell Biol.*

121, 387-395

Pleasure,S.J., Lee,V.M.-Y. and Nelson,D.L. (1990) *J. Neurosci.* **10**, 2428-2437

Sacher,M.G., Athlan,E.S. and Mushynski,W.E. (1992) *Biochem. Biophys. Res. Comm.* **186**, 524-530

Sacher,M.G., Athlan,E.S. and Mushynski,W.E. (1994) *J. Biol. Chem.* **269**, 14,480-14,484

Sharp,G.A., Shaw,G. and Weber,K. (1982) *Expl. Cell Res.* **137**, 403-413

Sihag,R.K. and Nixon,R.A. (1990) *J. Biol. Chem.* **265**, 4166-4171

Soifer,D., Nicoletti,V., Cabane,K., Mack,K. and Poulos,B. (1991) *J. Neurosci. Res.* **30**, 63-71

Steinert,P.M. and Roop,D.R. (1988) *Annu. Rev. Biochem.* **57**, 593-625

Swanstrom,R. and Shank,P.R. (1978) *Anal. Biochem.* **86**, 184-192

Tokutake,S., Liem,R.K.H. and Shelanski,M.L. (1984) *Biomed. Res.* **5**, 235-238

Weber,K. and Fuchs,E. (1994) *Annu. Rev. Biochem.* **63**, 345-382

Wong,P.C., and Cleveland,D.W. (1990) *J. Cell Biol.* **111**, 1987-2003

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Chapter 5

GENERAL DISCUSSION

Previous work had led to the conclusion that phosphate moieties on NF subunits were relatively stable. They were found to undergo turnover of up to 50% of the initially incorporated phosphates over a five day period during transport along the axon *in situ* (Nixon and Lewis, 1986). In addition Clark and Lee (1991) showed that in cultured sympathetic neurons a similar percentage of phosphates are turned over during a three day period. However, the demonstration that a small amount of phosphate is found in regions outside the tail domain of NF-M and NF-L (Sihag and Nixon, 1989,1990; Pleasure *et al*, 1990), indicated that more transient phosphorylation events were taking place.

In order to examine this possibility we chose to treat rat DRG cultures with the serine/threonine protein phosphatase inhibitor OA. Surprisingly, we noted differences in both the Triton solubility and phosphorylation state of NF subunits in treated as compared to untreated cultures within 30-60 min. (Chapter 2, fig.1; Chapters 3 and 4, figs.2). These results indicate that some phosphate moieties do indeed undergo rapid turnover and explain the earlier difficulty in identifying them against a high background of the more stable tail domain phosphorylation. Alternatively, OA may serve to increase protein kinase activity by interfering with regulatory mechanisms through the inhibition of PP-1 and PP-2A (Sassa *et al*, 1989).

The fact that phosphate moieties in the tail domain of both NF-M and NF-H turn over slowly may be explained by the results of several groups studying other systems. Agostinis *et al* (1990) and Donella-Deana *et al* (1990) reported that a phosphoserine residue was poorly dephosphorylated by the catalytic subunits of all four of the major serine/threonine protein phosphatases when it was followed by a proline residue. Subsequently, Xu *et al* (1992) showed that a phosphoserine residue carboxy-terminal to a lysine residue was poorly dephosphorylated by alkaline phosphatase. Taken together these results would indicate that a phosphoseryl sandwiched between an amino-terminal lysine residue and a carboxy-terminal proline residue (ie. Lys-Ser-Pro), as is found in multiple copies in the two

higher molecular weight NF subunits, would be more resistant to dephosphorylation. A sequence analysis of rat NF-M and NF-L showed that NF-L contained one serine residue preceded by a lysine residue within its head domain. In addition, while NF-L does not contain a serine residue followed by a proline residue, NF-M does contain two such sequences in its amino-terminal portion at amino acids-31 and -45. It would be of interest to determine whether these serines in NF-M are among the transiently phosphorylated residues seen following OA treatment as such phosphoserines would be expected to be relatively stable .

Further evidence for the presence on NF subunits of phosphate moieties which turn over rapidly stems from the observation that OA-induced Triton solubility was fully reversible for NF-L and NF-M within 2 hrs. of removing the inhibitor from the culture medium and within 10 hrs. for NF-H (Chapter 2, fig.3). The time frame for the reversibility of this OA-induced effect is much shorter than the turnover rate noted in previous studies (Nixon and Lewis, 1986; Clark and Lee, 1991).

We have shown directly for NF-L and indirectly for NF-M that the OA-induced phosphorylation of these subunits occurs predominantly in their amino-terminal head domain (Chapters 3 and 4, figs.2). The amino-terminal fragment of NF-M was too weakly ^{32}P-labeled following cleavage with NTCB to allow for two-dimensional phosphopeptide mapping. Although the C/N ratios for NF-M were not determined as they were for NF-L (cf. Chapter 3), they appeared to be extremely low indicating that the amino-terminal head domain was not highly phosphorylated to begin with as has been reported previously (Sihag and Nixon, 1990). In addition the low efficiency of NF-M cleavage obtained with NTCB further hindered our ability to obtain sufficient amounts of the head domain fragment for two-dimensional phosphopeptide mapping.

The correlation between NF fragmentation seen after 30-60 min. of OA treatment and the appearance of head domain phosphorylation in both NF-L and NF-M suggests that there may be a cause and effect relationship between the two events. This suggestion is consistent with observations that amino-terminal

phosphorylation of subunits in other IFs, including homopolymers composed of NF-L, caused their fragmentation both *in vitro* and *in vivo* (Inagaki *et al*, 1987,1988,1989,1990; Geisler and Weber, 1988; Ando *et al*, 1989; Geisler *et al*, 1989; Chou *et al*, 1990,1991; Heald and McKeon, 1990; Nishizawa *et al*, 1991).

A similar fragmentation of NFs was recently reported by Hisanaga *et al* (1994). These authors showed that reassembled NFs were broken into small, 100,000x*g* soluble oligomers following treatment with PKA. However, native NFs were more resistant to such treatment and soluble fragments were detected only at reduced centrifugation speeds. In addition, an unravelling of the PKA-treated filaments was observed by electron microscopy. Our results are more comparable with those obtained for the native NFs in the latter study and imply the involvement of PKA in the fragmentation of NFs seen *in vivo*. This suggestion conforms with the recent demonstration in our laboratory that PKA is involved in the appearance of Triton-soluble forms of all three NF subunits following OA treatment (B. Giasson and W.E. Mushynski, personal communication). Since PKA is known to phosphorylate the head domain of NF-L and NF-M (Sihag and Nixon, 1990; Hisanaga *et al*, 1994) this would imply that NF assembly/diassembly may be regulated by second messengers thus allowing for structural changes of the NF network in response to external stimuli. Direct testing of the cause and effect relationship suggested above hinges upon identification of the OA-induced phosphorylation sites in the head domain of both NF-L and NF-M. Point mutations of these residues could then be effected and the assembly of the mutant proteins could be examined both *in vitro* and *in vivo*.

The fragmentation of NFs seen at low OA concentrations and the reduction in gel electrophoretic mobility of all three NF subunits seen at higher OA concentrations (Chapter 3, fig.1) suggested that inhibition of PP-2A was responsible for the former while the added inhibition of PP-1 caused the latter. Similar treatment of BHK-21 cells with OA led to the suggestion that PP-1 was involved in maintaining the organization of the IF and MT networks in those cells (Eriksson

et al, 1992). The fact that PP-$2A_c$ but not PP-1_c could remove these OA-induced amino-terminal domain phosphates *in vitro* would appear to support the above suggestion concerning PP-2A (Chapter 3, fig.4). It would however be necessary to test the various forms of PP-2A (Chapter 1, table 2) for their ability to remove these OA-induced phosphates before conclusively implicating this enzyme in the regulation of NF dynamics since the various subunits seem to alter the activity and specificity of PP-2A (Imaoka *et al*, 1983; Tsuiki *et al*, 1985; Mumby *et al*, 1987; Chen *et al*, 1989; Sola *et al*, 1991; Goedert *et al*, 1992).

It is now well established that phosphorylation of NF-M and NF-H retards their gel electrophoretic mobility (Julien and Mushynski, 1982). However, the appearance of a slower migrating form of NF-L was never reported previous to our studies (Chapter 2, fig.1). More recently Hisanaga *et al* (1994) also reported the appearance of a slower migrating form of this subunit following *in vitro* phosphorylation although the mobility was not perturbed to the same degree as by OA. While it is not clear whether these slower migrating forms of NF-L arise from similar phosphorylation events it is noteworthy that both are found exclusively in the supernatant fraction following high speed centrifugation (>90,000xg), implying that highly phosphorylated forms of NF-L cannot assemble properly.

There are three possible explanations for the observation that both PP-1_c and PP-$2A_c$ caused the disappearance of the slower migrating forms of NF-L and NF-M. First, these results may simply be due to the use of unregulated catalytic subunits of the protein phosphatases. Once again use of the various forms of the protein phosphatases may provide further insight into their involvement in the generation of these electrophoretic variants. Alternatively, phosphate moieties acted on by either PP-1 or PP-2A may together be involved in generating these electrophoretic variants and removal of either type of phosphate will cause the disappearance of these slower migrating forms of the NF subunits. Finally, PP-1_c and PP-$2A_c$ may show overlapping specificity *in vitro* for the phosphate moieties responsible for producing these electrophoretic variants (Cohen, 1989). The

streptolysin-mediated introduction of I1, I2 or DARPP-32 into DRGs might provide some insight into whether inhibition of PP-1 alone is sufficient to produce these electrophoretic variants. We have shown that low OA concentrations cause the fragmentation of NFs but even prolonged treatment under these conditions did not cause a change in the electrophoretic mobility of any of the NF subunits (data not shown). Using a rationale similar to that stated above, this would suggest that inhibition of PP-2A alone is not sufficient to generate these electrophoretic variants.

Immunoprecipitation of OA-induced, Triton-soluble NF fragments under denaturing and non-denaturing conditions revealed that these soluble species are heterooligomers composed of NF-L associated with either NF-M or NF-H (Chapter 4). It has long been assumed that NF-L forms the core of the NFs (Sharp *et al*, 1982) as NF-L is the only subunit capable of forming long, 10 nm wide filaments *in vitro* (Geisler and Weber, 1981; Tokutake *et al*, 1984; Balin and Lee, 1991). This notion was bolstered by recent findings that NF-M and NF-H cannot co-assemble in an IF⁻ cell line (Ching and Liem, 1993), and that neurons from mutant quail which do not produce assembly competent NF-L do not contain IFs (Yamasaki *et al*, 1991). Lee *et al* (1993) showed that NF-M expressed to a level of just 10% of that of NF-L was sufficient to drive NF assembly in an IF⁻ cell line. This result is significant since it helps explain why both NF-L and NF-M are co-expressed during development with NF-H appearing later (Shaw and Weber, 1982; Pachter and Liem, 1984; Carden *et al*, 1987). In this respect it is interesting to note that filaments assembled *in vitro* from NF-L and NF-M were longer than those assembled from NF-L and NF-H (Balin and Lee, 1991).

Our data provide the first *in vivo* biochemical evidence that intermediates involved in the assembly and disassembly of NFs are composed of heterooligomers consisting of NF-L associated predominantly with either NF-M or NF-H. The existence of similar oligomers under normal conditions was shown by performing the same immunoprecipitation experiments with the Triton-soluble pool of NFs

from untreated DRG. Soluble NF oligomers have been reported previously in other systems (Shea *et al*, 1988; Gotow and Tanaka, 1994) and may represent the units that are incorporated along the length of the NFs (Okabe *et al*, 1993). The naturally occurring pool of Triton-soluble oligomers in DRGs differs somewhat from the OA-induced pool since the former can be pelleted at 100,000x*g* whereas the latter cannot after prolonged OA-treatment. In addition, the naturally occurring soluble pool contains small amounts of NF-M or NF-H bound to the NF-L/NF-H and NF-L/NF-M oligomers, respectively. The reason for this discrepancy is unclear, although it may simply reflect differences in the relative size distributions of the oligomers in the two soluble pools. Previously Carden and Eagles (1986) provided *in vitro* evidence for the existence of an NF-L/NF-M heterodimer following disassembly of ox NFs under low ionic strength and low pH conditions. However such a dimer was not detected at similar levels by cross-linking whole NFs.

Recently, Mulligan *et al* (1991) performed immunoelectron microscopic studies on reassembled NFs using monoclonal antibodies to known epitopes of the three NF subunits. These authors suggested that all reassembly and immunolabeling results could be explained if it was assumed that the basic building blocks of NFs were tetramers composed of homodimers of NF-L or heterodimers containing NF-L and either NF-M or NF-H. These authors suggested that heterodimers of NF-L/NF-M and NF-L/NF-H would be energetically more favourable than homodimers of NF-M or NF-H due to the relatively neutral tail domain of NF-L. In addition this model would predict a higher molar ratio of NF-L to both NF-M and NF-H as has been reported (Scott *et al*, 1985) and would imply that NF-H can be incorporated into pre-existing filaments as heterooligomers also containing NF-L.

Previously we speculated that NF subunits were released as homooligomers (Chapter 2). This was based upon the observed fragmentation and asynchronous Triton-solubilization of NF subunits following OA treatment. However, the further studies in Chapter 4 allowed us to conclude that the NF subunits are released and,

by implication, incorporated as heterooligomers. The most plausible explanation for the apparent asynchronous solubilization of the three NF subunits is to assume that the NFs are fragmenting along their lengths rather than across their widths. That is, since NF subunits (presumably in oligomeric states) are incorporated along the length of NFs (Angelides *et al*, 1989; Okabe *et al*, 1993) we can assume that NF disassembly would most easily be accomplished by a reversal of this process rather than by a severing of the NFs across their widths. This would explain the appearance of differing proportions of all three NF subunits in the Triton-soluble fraction. Of course, this model implies the existence of a gradient of each subunit across the diameter of NFs, with NF-H being more highly concentrated in the outer regions of the filament. NF-L would be found in these peripheral regions as well since it is required to permit the incorporation of NF-H. NF-L, however, would be more concentrated towards the core of the filaments since this subunit is expressed earlier in development than NF-H and a complete re-organization of the NF network following the appearance of NF-H has not been reported.

Our studies using the serine/threonine protein phosphatase inhibitor OA have allowed us to gain insight into the arrangement of the individual NF subunits within the NF. Besides providing *in vivo* biochemical evidence for the existence of heterooligomers of NF-L associated predominantly with either NF-M or NF-H we have also been able to show a correlation between amino-terminal domain phosphorylation of NF-L and NF-M and the fragmentation of NFs. The possibility remains that phosphorylation of the amino-terminal domain of NF subunits disrupts interactions between NFs and other cytoskeletal elements (Frappier *et al*, 1987; Hirokawa *et al*, 1988; Miyasaka *et al*, 1993) thus leading to Triton-solubility. However, it is unlikely that such an event was responsible for the OA-induced fragmentation since NFs reassembled in the absence of any other cytoskeletal component are insoluble at 13,000x*g*. It therefore appears that phosphorylation of the amino-terminal head domain of NF subunits regulates their assembly states as is the case with other IFs.

References

Agostinis,P., Goris,J., Pinna,L.A., Marchiori,F., Perich,J.W., Meyer,H.E. and Merlevede,W. (1990) *Eur. J. Biochem.* **189**, 235-241

Ando,S., Tanabe,K., Gonda,Y., Sato,C. and Inagaki,M. (1989) *Biochemistry* **28**, 2974-2979

Angelides,K.J., Smith,K.E. and Takeda,M. (1989) *J. Cell Biol.* **108**, 1495-1506

Balin,B.J. and Lee,V.M.-Y. (1991) *Brain Res.* **556**, 196-208

Carden,M.J. and Eagles,P.A.M. (1986) *Biochem. J.* **234**, 587-591

Carden,M.J., Trojanowski,J.Q., Schlaepfer,W.W. and Lee,V.M.-Y. (1987) *J. Neurosci.* **7**, 3489-3504

Chen,S.C., Kramer,G. and Hardesty,B. (1989) *J. Biol. Chem.* **264**, 7267-7275

Ching,G.Y. and Liem,R.K.H. (1993) *J. Cell Biol.* **122**, 1323-1335

Chou,Y.-H., Bischof,J.R., Beach,D. and Goldman,R.D. (1990) *Cell* **62**, 1063-1071

Chou,Y.-H., Ngai,K.-L. and Goldman,R.D. (1991) *J. Biol. Chem.* **266**, 7325-7328

Clark,E.A. and Lee,V.M.-Y. (1991) *J. Neurosci. Res.* **30**, 116-123

Cohen,P. (1989) *Annu. Rev. Biochem.* **58**, 453-508

Donella-Deana,A., McGowan,C.H., Cohen,P., Marchiori,F., Meyer,H.E. and

Pinna,L.A. (1990) *Biochim. Biophys. Acta* **1051**, 199-202

Eriksson,J.E., Brautigan,D.L., Vallee,R., Olmsted,J., Fujiki,H. and Goldman,R.D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11,093-11097

Frappier,T., Regnouf,F. and Pradel,L.A. (1987) *Eur. J. Biochem.* **169**, 651-657

Geisler,N. and Weber,K. (1981) *J. Mol. Biol.* **151**, 565-571

Geisler,N. and Weber,K. (1988) *EMBO J.* **7**, 15-20

Geisler,N., Hatzfeld,M. and Weber,K. (1989) *Eur. J. Biochem.* **183**, 441-447

Goedert,M., Cohen,E.S. and Cohen,P. (1992) *FEBS Lett.* **312**, 95-99

Gotow,T. and Tanaka,J. (1994) *J. Neurosci. Res.* **37**, 691-713

Heald,R. and McKeon,F. (1990) *Cell* **61**, 579-589

Hirokawa,N., Hisanaga,S.-I. and Shiomura,Y. (1988) *J. Neurosci.* **8**, 2769-2779

Hisanaga,S., Matsuoka,Y., Nishizawa,K., Saito,T., Inagaki,M. and Hirokawa,N. (1994) *Mol. Biol. Cell* **5**, 161-172

Imaoka,T., Imazu,M., Usui,H., Kinohara,N. and Takeda,M. (1983) *J. Biol. Chem.* **258**, 1526-1535

Inagaki,M., Nishi,Y., Nishizawa,K., Matsuyama,M. and Sato,C. (1987) *Nature* **328**, 649-652

Inagaki,M., Gonda,Y., Matsuyama,M., Nishizawa,K., Nishi,Y. and Sato,C. (1988) *J. Biol. Chem.* **263**, 5870-5978

Inagaki,M., Gonda,Y., Ando,S., Kitamura,S., Nishi,Y. and Sato,C. (1989) *Cell Struct. Funct.* **14**, 279-286

Inagaki,M., Gonda,Y., Nishizawa,K., Kitamura,S., Sato,C., Ando,S., Tanabe,K., Kikuchi,K., Tsuiki,S. and Nishi,Y. (1990) *J. Biol. Chem.* **265**, 4722-4729

Julien,J.-P. and Mushynski,W.E. (1982) *J. Biol. Chem.* **257**, 10,467-10,470

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

Miyasaka,H., Okabe,S., Ishiguro,K., Uchida,T. and Hirokawa,N. (1993) *J. Biol. Chem.* **268**, 22,695-22,702

Mulligan,L., Balin,B.J., Lee,V.M.-Y. and Ip,W. (1991) *J. Struct. Biol.* **106**, 145-160

Mumby,M.C., Russell,K.L., Garrard,L.J. and Green,D.D. (1987) *J. Biol. Chem.* **262**, 6257-6265

Nishizawa,K., Yano,T., Shibata,M., Ando,S., Saga,S., Takahashi,T. and Inagaki,M. (1991) *J. Biol. Chem.* **266**, 3074-3079

Nixon,R.A. and Lewis,S.E. (1986) *J. Biol. Chem.* **261**, 16,298-16,301

Okabe,S., Miyasaka,H. and Hirokawa,N. (1993) *J. Cell Biol.* **121**, 375-386

Pachter,J.S. and Liem,R.K.H. (1984) *Dev. Biol.* **103**, 200-210

Pleasure,S.J., Lee,V.M.-Y. and Nelson,D.L. (1990) *J. Neurosci.* **10**, 2428-2437

Sassa,T., Richter,W.W., Uda,N., Suganuma,M., Suguri,H., Yoshizawa,S., Hirota,M. and Fujiki,H. (1989) *Biochem. Biophys. Res. Comm.* **159**, 939-944

Scott,D., Smith,K.E., O'Brien,B.J. and Angelides,K.J. (1985) *J. Biol. Chem.* **260**, 10,736-10,747

Sharp,G.A., Shaw,G. and Weber,K. (1982) *Expl. Cell Res.* **137**, 403-413

Shaw,G. and Weber,K. (1982) *Nature* **298**, 277-279

Shea,T.B., Majocha,R.E., Marotta,C.A. and Nixon,R.A. (1988) *Neurosci. Lett.* **92**, 291-297

Sihag,R.K. and Nixon,R.A. (1989) *J. Biol. Chem.* **264**, 457-464

Sihag,R.K. and Nixon,R.A. (1990) *J. Biol. Chem.* **265**, 4166-4171

Sola,M.M., Langan,T. and Cohen,P. (1991) *Biochim. Biophys. Acta* **1094**, 211-216

Tokutake,S., Liem,R.K.H. and Shelanski,M.L. (1984) *Biomed. Res.* **5**, 235-238

Tsuiki,S., Kikuchi,K., Tamura,S., Hiraga,A. and Shneha,R. (1985) *Adv. Protein Phosphatases* **1**, 193-214

Xu,Z.-S., Liu,W.-S. and Willard,M.B. (1992) *J. Biol. Chem.* **267**, 4467-4471

Yamasaki,H., Itakura,C. and Mizutani,M. (1991) *Acta Neuropathol.* **82**, 427-434

Original contributions to knowledge

1. Treatment of primary cultures consisting of dissociated rat dorsal root ganglia with the serine/threonine protein phosphatase inhibitor okadaic acid (OA) was shown to:

a] cause a fragmentation of neurofilaments (NFs) as evidenced by the appearance of subunits in the Triton-soluble fraction and a disruption of the NF network as shown by immunofluorescence microscopy. The disruption of the NF network was manifested as a punctate staining of the axons.

b] an increase in amino-terminal domain phosphorylation of both NF-L and NF-M.

c] a reduction in the gel electrophoretic mobilities of all three NF subunits signifying an increase in carboxy-terminal domain phosphorylation.

2. Based on the response to varying OA concentrations fragmentation was inferred to be the result of protein phosphatase-2A inhibition whereas phosphorylation leading to a reduction in gel electrophoretic mobility was inferred to be due to inhibition of protein phosphatase-1.

3. Treatment of the OA-induced Triton-soluble NF fragments with either the catalytic subunit of protein phosphatase-1 ($PP\text{-}1_c$) or protein phosphatase-2A ($PP\text{-}2A_c$) showed that:

a] $PP\text{-}2A_c$ removed the OA-induced amino-terminal domain phosphate moieties in NF-L.

b] either $PP\text{-}1_c$ or $PP\text{-}2A_c$ was capable of removing phosphate moieties responsible for altering the gel electrophoretic mobilities of NF subunits.

4. Immunoprecipitation of Triton-soluble NFs demonstrated the existence of heterooligomers consisting of NF-L associated predominantly with either NF-M or NF-H. These oligomers were present in the 100,000xgTriton supernatant and may represent intermediates involved in the dynamic assembly and disassembly of NFs.