PHOSPHORYLATION OF NEUROFILAMENT PROTEINS

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ABSTRACT

This chapter provides an overview of neurofilament structure and phosphorylation, with major emphasis on protein kinases and protein phosphatases involved in the modification of neurofilament proteins. Recent advances in the identification of protein kinases that act on neurofilaments can now be complemented by studies on protein phosphatases due to the availability of highly potent and specific phosphatase

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inhibitors such as okadaic acid. We show that treatment of cultured dorsal root ganglion neurons with okadaic acid caused a shift in the electrophoretic mobilities of neurofilament subunits, signifying their increased phosphorylation. This was accompanied by the ordered conversion of neurofilament subunits to Triton X-100-soluble forms and by marked changes in their immunofluorescent staining patterns. These results indicate that neurofilaments are dynamic entities whose assembly and organization are modulated by the balanced addition and removal of phosphate moieties.

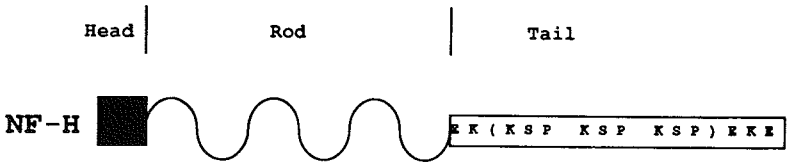
I. INTRODUCTION

Neurofilaments (NFs) belong to a class of cytoskeleton components known as intermediate filaments. Intermediate filaments have a diameter of approximately 10 nm, and their protein subunits can be divided into five subclasses on the basis of sequence comparisons (see Steinert and Roop, 1988, for a review). The expression of these different proteins is characterized by high levels of tissue specificity (Osborn and Weber, 1982). NFs represent the principal intermediate filament found in neurons. Other neuron-specific intermediate filament proteins such as peripherin (Portier et al., 1984; Parysek et al., 1988), α-internexin (Fleigner et al., 1990), and nestin (Lendahl et al., 1990) are either limited in distribution or appear transiently during development.

In common with other intermediate filament proteins, NF subunits contain a highly conserved α-helical "rod" domain, which is flanked on one side by the N-terminal "head" domain and on the other by the C-terminal "tail" domain (Geisler et al., 1983). This domain organization is illustrated for one of the NF subunits in Figure 1. The biochemical and antigenic differences between intermediate filament proteins from different subclasses are to a considerable degree due to the variable head and tail domains (Steinert and Roop, 1988). The formation of filaments of similar diameter and morphology by the different proteins reflects involvement of the highly conserved rod domain, which enables different intermediate filament subunits to co-assemble in cells transfected with heterologous cDNAs (Monteiro and Cleveland, 1989; Chin and Liem, 1990).

Mammalian NFs are composed of three subunits with apparent molecular masses of about 68 kDa (NF-L), 150 kDa (NF-M), and 200 kDa (NF-H), as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Hoffman and Lasek, 1975). The latter size estimates were shown to be too high because of the anomalous behavior of NF proteins on SDS-PAGE (Kaufmann et al., 1984; Georges and Mushynski, 1987). Actual values for NF-L, NF-M, and NF-H, based on sequencing data, are about 62 kDa (Geisler et al., 1985), 100 kDa (Myers et al., 1987), and 115 kDa (Julien et al., 1988), respectively.

The size differences between the three NF subunits are largely due to variations in the length of their C-terminal tail domains, which are rich in charged amino acids (Geisler et al., 1983; Julien and Mushynski, 1983) and can be seen to project from



Source of NF-H	Number of KSP Repeats	Most Abundant Repeat Unit(s)	Reference
Human	43	KSPEKAKSP$^{V}_{A}$KEEA	Lees et al., 1988
Mouse	51	KSP$^{G}_{A}$EA	Julien et al., 1988
Rat	52	KSPAE$^{V}_{A}$	Chin and Liem, 1990

Figure 1. Domain structure of NF-H and consensus sequences of the most abundant repeated motifs in the tail. The amino acid sequences are listed using the single letter symbols: A, Ala; E, Glu; G, Gly; K, Lys; P, Pro; S, Ser; V, Val. The amino acid symbols depicted in the tail portion of the diagram of NF-H emphasize the high levels of these residues in the domain.

the filament axis in rotary shadowed samples of NFs (Hisanaga and Hirokawa, 1988). The tail domain of NF-H forms apparent cross-bridges between NFs and other structures (Hirokawa et al., 1984), but the nature and role of these apparent contacts are still unknown.

Transfection studies with variously deleted NF subunit-encoding cDNAs have indicated that sequences in the head domain are required for early steps in filament assembly. Deletions within the C-terminal region of the rod domain yield mutant proteins whose incorporation into intermediate filaments in small amounts cause their rapid disruption (Wong and Cleveland, 1990; Gill et al., 1990). The latter effect indicates that NFs are dynamic entities capable of rapid subunit exchange (Angelides et al., 1989; Steinert and Liem, 1990) rather than the stable, unchanging structures they were once thought to be (Lasek et al., 1983). The presence of soluble forms of NF subunits in cells may in part reflect this exchange process (Georges et al., 1987; Lindenbaum et al., 1987).

II. NEUROFILAMENT PHOSPHORYLATION

A. General Background

The NF subunits from rat myelinated axons contain, on average, about 3, 6, and 14 moles of phosphate per mole of NF-L, NF-M, and NF-H, respectively. These

numbers are lower than the initially reported values (Julien and Mushynski, 1982), because they have been calculated using the true molecular weights of NF subunits which became available more recently (see above). Subsequent reports showed similar levels of phosphate in bovine (Wong et al., 1984), porcine (Georges et al., 1986), and human NFs (Ksiezak-Reding and Yen, 1987), although bovine NFs have also been reported to contain much higher amounts of phosphate (Jones and Williams,1982; Ksiezak-Reding and Yen, 1987). Treatment of NFs with alkaline phosphatase causes an increase in the mobilities of NF-M and NF-H on SDS-PAGE (Julien and Mushynski, 1982). This effect of phosphorylation on the gel electrophoretic mobilities of NF-M and NF-H provides a simple means of assessing the approximate phosphorylation states of NF-M and NF-H, keeping in mind that the subunits from exhaustively dephosphorylated NFs still contain significant levels of phosphate (Georges et al., 1986; Ksiezak-Reding and Yen, 1987).

The analysis of proteolytic cleavage products of NFs showed that the phosphate moieties in NF-M and NF-H were for the most part located in the tail domain (Julien and Mushynski, 1983), and ^{31}P-NMR analysis verified that those in NF-H were clustered (Zimmerman and Schlaepfer, 1986). The sequence characteristics of the phosphorylated region were reported by two groups in 1987. Myers et al. (1987) showed that the tail domain of human NF-M contains six tandem repeats of a 13 amino acid sequence, each containing two copies of the sequence Lys-Ser-Pro (henceforth referred to as KSP, using the single letter abbreviations for these amino acids). Geisler et al. (1987) reported that the tail domains of porcine NF-M and NF-H contained degenerate repetitive sequences with the KSP motif. The Ser in this repeated triplet represents the major phosphorylation site in NF-M and NF-H (Geisler et al., 1987; Lee, V.M.-Y. et al., 1988; Xu et al., 1992). Mouse (Levy et al., 1987) and rat (Napolitano et al., 1987) NF-M each contain only five copies of the KSP sequence, and these are not clustered to the degree seen in human NF-M. As indicated in Figure 1, the KSP triplet is repeated 51 times in the tail domain of mouse NF-H (Julien et al., 1988), 52 times in rat NF-H (Chin and Liem, 1990), and 43 times in human NF-H (Lees et al., 1988).

The KSP repeats are highly conserved in large NF proteins (Lee et al., 1986; Mencarelli et al., 1991), although there is some interspecies variation in the length and sequence of the spacers that separate this triplet sequence (Fig. 1; also see Geisler et al., 1987). A survey of available protein sequences indicates that the KSP motif is comparatively rare. Next to the large NF subunits, KS/TP occurs most prominently in histone H1 (Suzuki, 1989). MAP-2 and tau each contain two KSP sequences (Lee, G., et al., 1988; Lewis et al., 1988), whereas nestin contains one (Lendahl et al., 1990). There appears to be a redundancy of KSP phosphorylation motifs in NF-H, as less than one-third of the sites are modified in highly phosphorylated NF-H from rat myelinated axons (Julien and Mushynski, 1982; Chin and Liem, 1990).

The discovery of monoclonal antibodies that could distinguish between phosphorylated and dephosphorylated forms of NF-H and NF-M (Sternberger and

Sternberger, 1983) represented a major breakthrough in NF research. These antibodies, which recognize KSP repeat domains (Lee, V. M.-Y., et al., 1988), provide a facile means of monitoring the phosphorylation states of NF-M and NF-H *in situ* or by Western blot analysis. Their use has shown that the NF-H and NF-M in axons are generally more highly phosphorylated than those in the perikaryon and dendrites, although certain pathological states are associated with increased phosphorylation of perikaryal NFs (Schlaepfer, 1987). The extensive library of monoclonal antibodies characterized by Lee and co-workers provides unprecedented levels of discrimination between a broad range of phosphorylation states (Carden et al., 1985; Lee et al., 1987). Some of the antibodies can also be used to estimate the spacing between phosphate moieties (Clark and Lee, 1991b).

The structural organization of NFs is generally regarded as consisting of a central, thread-like core composed of associated rod domains, with a peripheral zone of highly charged lateral projections representing the tail domains of NF subunits (Julien and Mushynski, 1983; Weber et al., 1983; Hisanaga and Hirokawa, 1988). The multiple phosphorylation sites in the tail domains are important elements of this charged peripheral zone. Their capacity to undergo cyclical phosphorylation/dephosphorylation (Nixon and Lewis, 1986) represents a potential mechanism for modulating conformational changes (Otvos et al., 1988) relevant to tail domain function(s). The enzymes that mediate these cyclical changes are therefore of utmost relevance to any discussion of NF phosphorylation.

B. Protein Kinases

A number of protein kinase activities copurify with mammalian NFs and/or phosphorylate NF subunits *in vitro* (see Table 1). Although some of these associations may be physiologically relevant, artefactual binding of kinases to the highly charged NF proteins might also occur. Additional information is therefore required to establish whether a protein kinase phosphorylates NFs *in vivo*.

The major activity copurifying with NFs is regulator-independent and shares some properties with casein kinase I (Julien et al., 1983). A similar protein kinase is associated with squid NFs (Floyd et al., 1991), indicating that the relationship is conserved in nerve cell evolution. The regulator-independent protein kinase was implicated in the *in vivo* phosphorylation of NFs by comparative mapping of phosphopeptides from *in vivo* and *in vitro* ^{32}P-labeled NF subunits (Julien and Mushynski, 1981).

Fractionation of the regulator-independent activity yielded a so-called NF kinase that also phosphorylated other proteins (Toru-Delbauffe et al., 1986) as well as a NF-specific kinase that phosphorylated the tail domain of NF-H exclusively (Wible et al., 1989). The latter enzyme did not act on dephosphorylated NF-H, indicating that prior phosphorylation of the substrate is required.

Protein kinase A and protein kinase C also copurify with NFs and have been shown to phosphorylate NF subunits *in vitro*. The preferred substrate for the two

Table 1. Protein Kinases That Phosphorylate Mammalian Neurofilament Proteins

Type Protein Kinase	*Copurifies with NFS*	*NF Phosphorylation*		*Preferred Substrate*	*References*
		In Vitro	*In Vivo*		
Regulator-independent	+	+	Mapping	M > L > H	Julien and Mushynski, 1981; Runge et al., 1981; Shecket and Lasek, 1982; Julien et al., 1983
NF kinase	+	+	ND	H > M > L	Toru-Delbauffe et al., 1986
	+	+	ND	H	Wible et al., 1989
	–	+	ND	KSP sites	Roder and Ingram, 1991
Protein kinase A	ND	+	–	M >> H, L	Leterrier et al., 1981
	+	ND	ND	Peptide	Caputo et al., 1989; Dosemeci et al., 1990; Dosemeci and Pant, 1992
	ND	+	Mapping	L, M head	Sihag and Nixon, 1989, 1990
Protein kinase C	ND	+	Mapping	M > L > H	Sihag et al., 1988
	+			M head	Sihag and Nixon, 1990
		ND	ND	Peptide	Dosemeci et al., 1990
	ND	ND	Phorbol	M > L > > H	Georges et al., 1989; Clark and Lee, 1991a
Ca^{2+}/Calmodulin kinase II	+	+	ND	M > L > H	Vallano et al., 1985
	+	+	Mapping	L	Sihag and Nixon, 1989
	+	ND	ND	Peptide	Caputo et al., 1989; Dosemeci et al., 1990
cdc2 kinase	ND	+	ND	KSP sites	Hisanaga et al., 1991; Guan et al., 1992
Protein kinase FA	ND	+	ND	M > L, H	Guan et al., 1991

Notes: "Copurifies with NFs" denotes protein kinases found in NF-enriched fractions (+). Involvement in the *in vivo* phosphorylation of NFs determined by comparative phosphopeptide mapping (mapping) of *in vivo* and *in vitro* ^{32}P-labeled NF subunits or by activation of protein kinase C (phorbol ester) in metabolically labeled cell cultures. Under the heading "preferred substrate," L, M, H refer to the NF subunits, "head" and "tail" refer to the N- and C-terminal domains, "peptide" refers to cases where kinase-specific peptide substrates were employed, "KSP sites" refers to the repeats in the tail domains of NF-M and NF-H. ND, not determined.

enzymes is NF-M (Table 1). Sihag and Nixon (1989, 1990) demonstrated by comparative phosphopeptide mapping that the two kinases phosphorylate sites in the head domain of NF-M and NF-L while the regulator-independent kinase phosphorylates sites in the tail domain. This work led to the proposal that head and tail domain phosphorylations are under separate regulation. The *in vivo* phosphorylation of NF subunits by protein kinase C has also received more direct support from studies showing that phorbol esters stimulate NF phosphorylation in cultured cells (Georges et al., 1989; Clark and Lee, 1991a).

The role of Ca^{2+}/calmodulin kinase II in NF phosphorylation is unclear at present, although comparative phosphopeptide mapping suggests that it may be involved

(Sihag and Nixon, 1989). The rationale for studying the *in vitro* phosphorylation of NF proteins by protein kinase F_A (Guan et al., 1991) is based on its involvement in the sequential phosphorylation of glycogen synthase. This process, also known as hierarchical phosphorylation (Roach, 1991), involves the creation of a phosphorylated recognition site by a protein kinase, followed by one or more phosphorylations by a second kinase that requires such phosphorylated sites in the substrate protein. Multiply phosphorylated proteins such as NF-M and NF-H are potential candidates for this type of interdependent phosphorylation. It is noteworthy that the NF kinase isolated by Wible et al. (1989) appeared to require prior phosphorylation of the tail domain, perhaps signifying the participation of a second enzyme in phosphorylation of the KSP repeats. A potential candidate for this role might be a protein kinase related to cdc2 kinase.

The cdc2 kinases from starfish oocytes (Hisanaga et al., 1991) and mammalian cells (Guan et al., 1992) were shown to phosphorylate the tail domain of dephosphorylated NF-H. The addition of only four phosphates per polypeptide to dephosphorylated bovine NF-H by the starfish enzyme was sufficient to return its electrophoretic mobility to the position of fully phosphorylated NF-H. Since it appeared that the less abundant KSPXK motif was phosphorylated by the starfish cdc2 kinase (Hisanaga et al., 1991), phosphorylation of the KSP repeat may involve a hierarchical type of mechanism.

The NF kinases purified by Roder and Ingram (1991) appear to phosphorylate the KSP repeats in exhaustively dephosphorylated NF-H and NF-M as they restore the phosphorylated epitopes recognized by the monoclonal antibody SMI 31 (Sternberger and Sternberger, 1983). However, one of these activities (PK40) phosphorylated NF-M at a much higher stoichiometry than NF-H, achieving only a partial shift in the mobility of the latter subunit. The second kinase (PK36) phosphorylated NF-H very poorly. These results suggest again that phosphorylation of the KSP repeats may involve more than one enzyme.

It is not known at present whether the enzyme(s) involved in phosphorylating KSP sites in NF-M and NF-H is (are) found exclusively in neurons. Transfected NF-H expressed in fibroblasts is not phosphorylated (Chin and Liem, 1990; Nash and Carden, 1991), whereas transfected NF-M is (Chin and Liem, 1990). Human NF-M, which contains multiple tandem repeats of the KSP sequence similar to but not as extensive as those seen in NF-H, is phosphorylated at these sites in transfected mouse L cells (Pleasure et al., 1990). Clearly, more work is required to explain these seemingly disparate results.

C. Protein Phosphatases

The preceding section has provided an overview of the extensive literature on NF phosphorylation by NF-associated and exogenous protein kinases. In marked contrast, little is known about the protein phosphatase(s) that act on NF proteins *in vivo*, as exemplified by the lack of information on this subject in reviews of NF

phosphorylation that have appeared within the past 5 years (Schlaepfer, 1987; Matus, 1988; Nixon and Sihag, 1991). Although phosphate turnover in NF proteins has been reported in a number of systems (e.g., Nixon and Lewis, 1986; Georges et al., 1987; Lindenbaum et al., 1987), a survey of the recent literature turned up only a few reports on NF-associated phosphatase activity (Guru et al., 1991; Shetty et al., 1992).

Phosphate turnover reflects the combined actions of protein kinases and phosphatases, the relative activities of the two types of enzyme determining the phosphorylation state of NF proteins. The recent report that okadaic acid (OA) increases vimentin phosphorylation in fibroblasts (Yatsunami et al., 1991) prompted us to study its effect on NF proteins. OA is a specific and highly potent inhibitor of protein phosphatase 1 (PP-1) and protein phosphatase 2A (PP-2A) (Bialojan and Takai, 1988). This toxin is a complex fatty acid derivative made by dinoflagellates, and its hydrophobicity allows it to be used on intact cells to identify physiological substrates of PP-1 and PP-2A (see Hardie et al., 1991, for a review).

The system we selected to study the effects of OA on NF phosphorylation consisted of primary cultures of dissociated dorsal root ganglia (DRG) from E15 rat embryos (Windebank et al., 1985) maintained in a defined medium (Bottenstein and Sato, 1979). Our previous work has shown that neurons in these cultures express high levels of all three NF subunits and that NF-M and NF-H are present in a broad range of phosphorylation states.

Different concentrations of OA were tested for their effects on the behavior of NF subunits on SDS-PAGE, as increased phosphorylation of the KSP repeats in the tail domains of NF-M and NF-H is known to reduce the electrophoretic mobilities of the subunits (Julien and Mushynski, 1982). Figure 2 shows that a reduction in the mobility of the most highly phosphorylated form of NF-H (pH) occurred after the DRG neurons were exposed to 10 nM OA for 6 hours. On the other hand, the mobility of the hypophosphorylated form of NF-H (H) did not shift noticeably until 100 nM OA and then continued to decrease at higher OA concentrations. The mobility shift of the highly phosphorylated form of NF-H at 10 nM OA was unexpected because the concentrations of PP-1 and PP-2A in most cells approach the micromolar range (Hardie et al., 1991). This result may indicate that phosphate turnover on highly phosphorylated NF-H is more rapid than on the hypophosphorylated form, or that more phosphates must be added to the latter to obtain an observable shift in mobility. However, *in vitro* studies with a cdc2 kinase have shown that the maximal shift in mobility of dephosphorylated NF-H is observed after phosphorylation of only four of the repeated KSP sequences (Hisanaga et al., 1991). This *in vivo* response to 10 nM OA indicates that PP-2A may be involved in dephosphorylation of the NF-H tail domain, because it is more sensitive to OA inhibition ($IC_{50} \approx 0.1$ nM) than is PP-1 ($IC_{50} \approx$ (10–15) nM) (Cohen, 1991). In addition, PP-2A has been shown to dephosphorylate the KS/TP sites in histone H1 that are phosphorylated by cdc2 kinase (Sola et al., 1991).

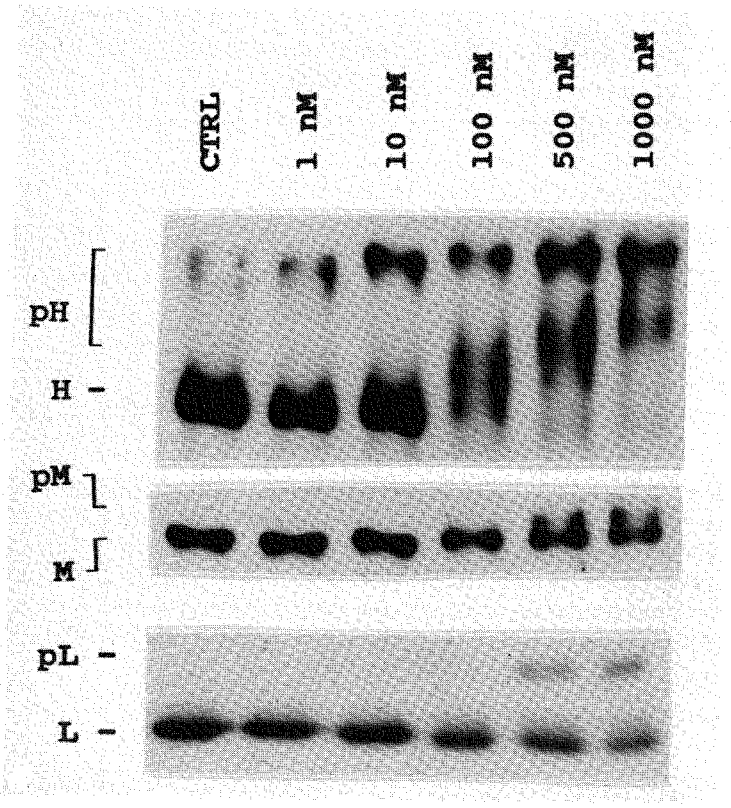


Figure 2. Effect of different okadaic acid concentrations on neurofilament subunits in dorsal root ganglion neurons. DRG cultures were treated for 6 hours with increasing concentrations of OA. Total protein was fractionated on a 5% SDS-polyacrylamide slab gel, transferred to polyvinylidene fluoride (PVDF) membrane, and detected using specific antibodies to the three NF subunits obtained from Sigma. H, M, and L refer to the NF subunits containing low phosphate levels, and pH, pM, and pL refer to the more highly phosphorylated forms of the subunits.

The appearance of slower migrating forms of NF-M (pM) and NF-L (pL) was observed at 500 nM OA and persisted at 1000 nM OA (Fig. 2). Similar to the situation for NF-H, the effect of phosphorylation on the gel electrophoretic mobility of NF-M could be due to modification of the KSP repeats in the tail domain. On the other hand, NF-L does not contain similar KSP sequences and the nature of other sites whose phosphorylation would reduce the mobility of the subunit is unknown. As shown by two-dimensional gel electrophoresis (Fig. 3), the mobility changes were accompanied by decreases in the isoelectric points of NF-M and NF-L to more acidic values, because of increased phosphorylation.

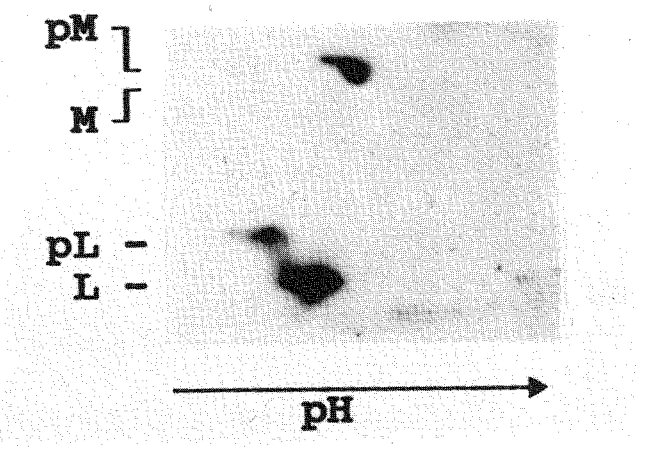


Figure 3. Two-dimensional gel electrophoresis of dorsal root ganglion cultures treated with okadaic acid. DRG cultures were treated with 1000 nM OA for 6 hours, harvested in 9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, and ampholytes in the pH range 5–8, focused in tube gels in the first dimension; and fractionated on a 5% SDS-polyacrylamide slab gel in the second. Proteins were transferred to PVDF membrane and detected with antibodies against NF-L and NF-M. The subunits are designated as described in the legend to Figure 1.

We chose to use OA at 1000 nM in all subsequent experiments since this concentration has been recommended for studies with intact cells because of their high levels of PP-1 and PP-2A (Hardie et al., 1991). Since *in vitro* phosphorylation of the head domain of NF-L homopolymer has been shown to block its assembly and cause disruption of NF-L (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990), we determined whether the change in phosphorylation state of NF proteins that accompanies OA treatment affected their state of assembly. This was accomplished by extracting OA-treated cells in Triton X-100-containing buffer and centrifuging to separate the detergent-insoluble cytoskeleton from soluble subunits. In control cells, all three NF subunits were found in the cytoskeleton fraction. Within half an hour almost all of the NF-H appeared in the supernatant fraction along with smaller proportions of NF-M and NF-L. By 3 hours all of the NF-M was redistributed to the supernatant fraction, whereas complete disappearance of NF-L from the pellet fraction was seen at 6 hours. The entire process was completely reversed within 24 hours of removing OA from the cultures. The order in which the NF subunits reassociated with the cytoskeletal fraction after removal of OA was the exact reverse of their order of dissociation (Sacher et al., 1992).

Under the centrifugation conditions we had used to separate Triton-soluble and -insoluble components, the soluble fraction has been shown to contain oligomeric NF proteins in addition to the subunits (Shea et al., 1988). Since the order of appearance of NF subunits in the Triton-soluble fraction progressed from NF-H to NF-M and finally NF-L, it is unlikely that the OA-induced conversion to soluble forms was due to the simple fragmentation of NFs. It is interesting to note that the dissociation pattern conforms with models of NF organization in which NF-L is proposed to form the filament core, whereas NF-M and NF-H have a peripheral localization (see Tokutake, 1990, for a review). This sequential solubilization of NF subunits should be accounted for in any model of heteropolymeric NF assembly, because it implies that the different subunits may be incorporated as separate units.

The shift of NF subunits to Triton X-100-soluble forms in OA-treated cultures indicated that the NF network was being disrupted. To verify this point, pairs of cultures were treated with OA for up to 6 hours, and one member of each pair was harvested for Western blot analysis while the other was fixed for immunofluores-

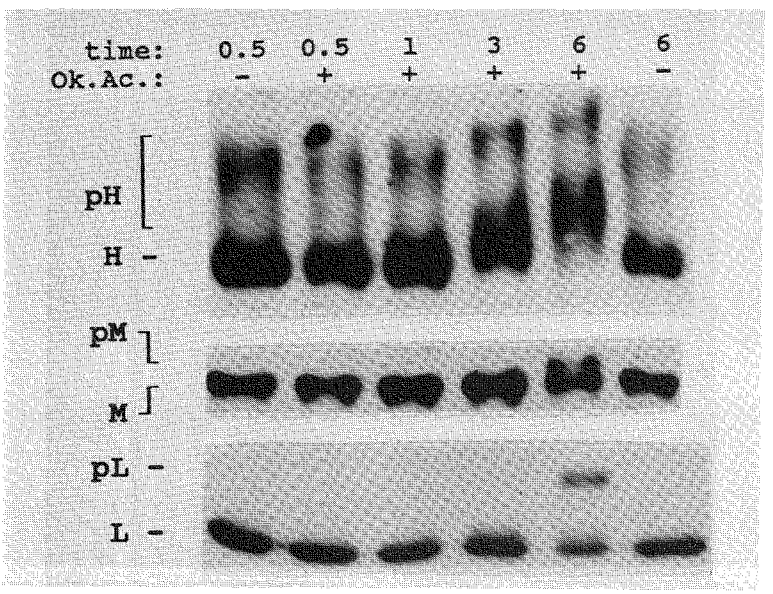


Figure 4. Time course of okadaic acid treatment of dorsal root ganglion cultures analyzed by Western blotting. DRG cultures were treated with 1000 nM OA (+) or without the inhibitor (–) for the stated amount of time. Total protein was fractionated on a 5% SDS-polyacrylamide gel, transferred to PVDF membrane, and detected using antibodies specific to each NF subunit. Sister cultures were used for immunofluorescence staining (see Fig. 5). The NF subunits are designated as described in the legend to Figure 1.

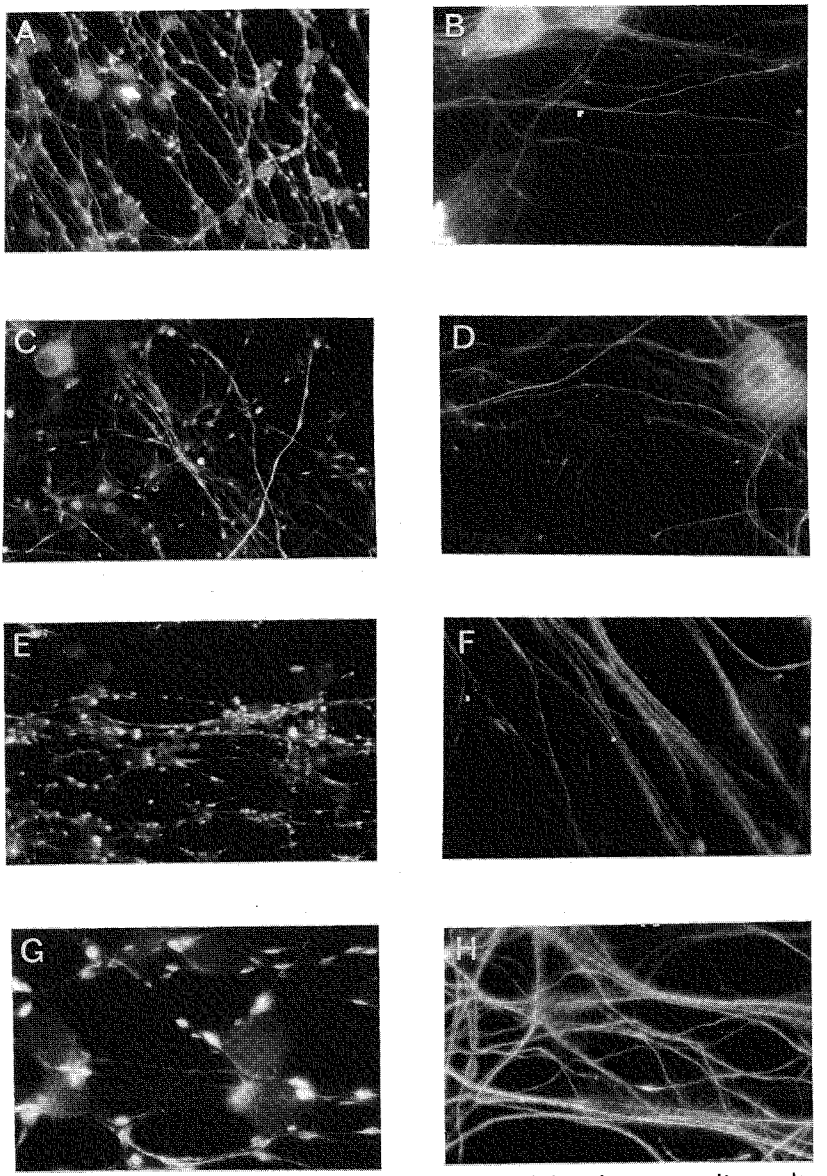


Figure 5. Time course of okadaic acid treatment of dorsal root ganglion cultures analyzed by immunofluorescence microscopy. DRG cultures were treated with OA for 0.5 hour (**A**, **C**, **E**, and **G**) or grown in its absence, fixed with ethanol:acetic acid (90:10), blocked, and stained with SMI31 (**A**, **B**), anti-H (**C**, **D**), anti-M (**E**, **F**), and anti-L (**G**, **H**) antibodies followed by Texas Red conjugated to an anti-mouse secondary antibody.

cence microscopy. As can be seen from the Western blot in Figure 4, NF-L and NF-M increased in apparent molecular weight only after 6 hours, whereas the faster migrating form of NF–H (H) began to shift within 1 hour of OA treatment. Although no changes in the mobilities of NF subunits were seen after 0.5 hours of OA treatment, antibodies to each NF subunit showed marked changes in the immunofluorescence staining pattern (Fig. 5, A, C, E, and G). The continuous axonal staining of control neurons differed markedly from the punctate staining of axons in the OA-treated cells. This punctate staining did not indicate the extent of NF disruption. Electron microscopy of cultures treated with OA for 30 minutes showed that intermediate filaments were still present in neurites, although they were somewhat scarcer than in controls (Fig. 6). The presence of visible intermediate filaments (some, no doubt, consisting of NFs) after 30 minutes of OA treatment is not surprising, considering the fact that significant amounts of only NF-H had been rendered Triton X-100-soluble at this time (Sacher et al., 1992).

Since the preceding results indicated that OA was increasing the phosphorylation state of NF proteins, we wished to confirm that the toxin was affecting phosphate turnover. DRG cultures were metabolically labelled with $^{32}P_i$, washed, and placed in $^{32}P_i$-free medium. OA was added, and samples were harvested at various times and immunoprecipitated with antisera against NF-M and NF-H. Figure 7 shows that in control cells, ^{32}P turnover could be observed over the 6-hour chase period for NF-M and NF-H. The particular rabbit polyclonal anti-NF-H antiserum used in this experiment immunoprecipitated only the highly phosphorylated form of NF-H (pH, cf. Fig. 2). ^{32}P turnover in NF-M and NF-H was essentially halted in OA-treated cells. ^{32}P labeling of the subunits actually appeared to increase during the chase period, as indicated by both increased ^{32}P content and reduced mobility on SDS-PAGE (Fig. 7).

The continued labeling of NF-H in particular could indicate that OA is exerting an effect not only through inhibition of PP-2A and/or PP-1, but also by activating protein kinase(s) that phosphorylates the subunits. A type 2A phosphatase is known to negatively regulate cdc2 kinase (Yamashita et al., 1990; Félix et al., 1990), and *in vitro* studies suggest that a related type of protein kinase may be involved in phosphorylating the KSP repeats in the tail domains of NF-H and NF-M. (Hisanaga et al., 1991; Guan et al., 1992).

The present study indicates that phosphate turnover is taking place on both the head and tail domains of NF subunits. Inhibition of head domain dephosphorylation would account for the disassembly of NFs, whereas hyperphosphorylation of the tail domain would cause the reduced mobility of NF subunits on SDS-PAGE. Although evidence has been presented that head and tail domain phosphorylation are under separate regulation (Nixon and Sihag, 1991), dephosphorylation of the two domains may be mediated by the same enzyme, possibly PP-2A.

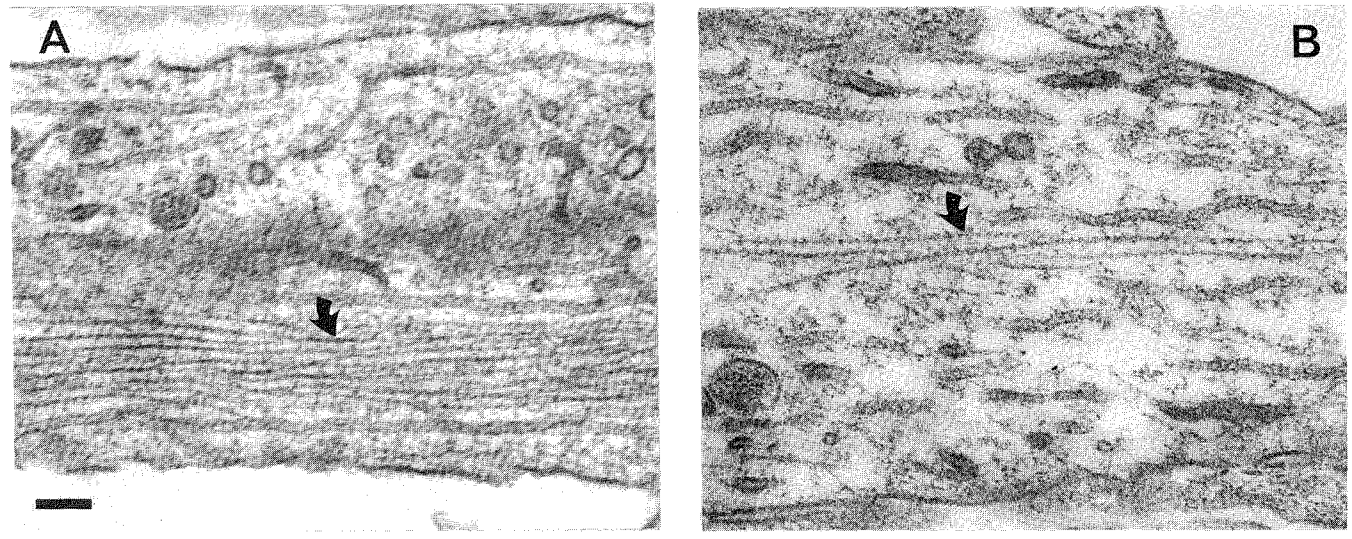


Figure 6. Electron micrographs of neurites from control (**A**) and okadaic acid-treated (**B**) cultures. The curved arrow points to intermediate filaments (presumably NFs) in the two samples. The intermediate filament bundles in neurites from cultures treated with OA for 30 minutes (**B**) were for the most part much sparser than those in controls (**A**). The bar represents 100 nm.

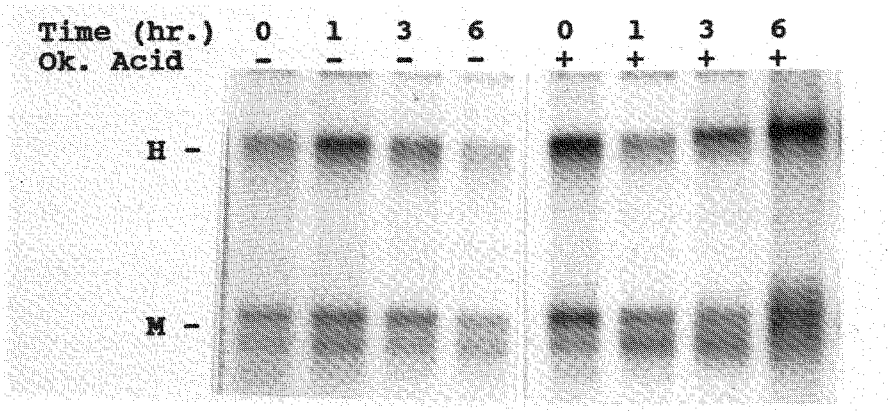


Figure 7. Immunoprecipitation of dorsal ganglion cultures labeled with $^{32}P_i$ and chased in the presence of okadaic acid. DRG cultures were labeled overnight with 500 μCi/ml carrier-free $^{32}P_i$, washed with unlabeled medium, and incubated with (+) or without (–) 1000 OA for the times shown prior to immunoprecipitation with antibodies specific for the three subunits. NF-H, NF-M, and NF-L refer to the locations of rat NF standards.

III. THE ROLE OF NEUROFILAMENT PHOSPHORYLATION

The finding that the head and tail domains of NF subunits are phosphorylated by different protein kinases (Sihag and Nixon, 1989, 1990) suggests that phosphorylation modulates several aspects of NF metabolism. The dephosphorylation of NF subunits by alkaline phosphatase does not affect their *in vitro* assembly (Georges et al., 1986), indicating that the phosphate moieties in isolated NFs are not involved in filament formation. These phosphates are located mainly in the tail domain of the subunits (Julien and Mushynski, 1983). On the other hand, *in vitro* phosphorylation of sites in the head domain of NF-L by protein kinase A or protein kinase C inhibits NF-L assembly and initiates filament disassembly (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990). This effect of head domain phosphorylation has also been observed for other intermediate filament proteins and may represent a general mechanism for regulating their assembly (see Skali and Goldman, 1991, for a review). The disassembly of NFs to Triton-soluble components in OA-treated neurons (see above) suggests that this type of mechanism may operate *in vivo*, although the location of phosphorylation sites in the soluble subunits remains to be determined.

Another role for phosphorylation may be to stabilize NF proteins. *In vitro* studies have demonstrated that dephosphorylation renders NF subunits, and NF-H in particular, more susceptible to proteolysis (Goldstein et al., 1987; Pant, 1988). The increased phosphorylation of NF-H in nerve growth factor-treated PC12 cells might

explain how NF-H levels can increase in these cells without there being a change in the steady-state level of NF-H mRNA (Lindenbaum et al., 1987, 1988).

Phosphorylation has been shown to affect both homologous and heterologous NF interactions. The capacity of NFs to form a reticulated network *in vitro* is abolished by dephosphorylation and reestablished by protein kinase A treatment (Eyer and Leterrier, 1988). Dephosphorylation of NF-H has been reported to block its ability to stimulate tubulin polymerization (Minami and Sakai, 1985) and to promote its association with taxol-stabilized microtubules (Hisanaga and Hirokawa, 1990). The reason for these contradictory results is unknown, although Hisanaga and Hirokawa (1990) found that neither phosphorylated nor dephosphorylated NF-H had any effect on tubulin polymerization. More recently, Hisanaga et al. (1991) showed that inhibition of the binding of NF-H to microtubules could be restored when the dephosphorylated subunit was treated with cdc2 kinase. This indicates that the KSP repeat domain is involved in microtubule binding.

The effect of dephosphorylation on the gel electrophoretic mobilities of NF-H and MF-M (Julien and Mushynski, 1982) may be due at least partly to conformational changes. Studies with synthetic peptides corresponding to segments of the KSP repeat domain of human NF-M showed marked conformational differences between the phosphorylated and unphosphorylated species (Otvos et al., 1988). However, structural changes could not be detected in electron microscopic studies, which showed that inhibition of the phosphate moieties had no effect on the appearance of lateral projections, or on the ability of NFs to form cross-bridges *in vitro* (Hisanaga and Hirokawa, 1989).

The proposal that NFs are determinants of axonal caliber (Hoffman et al., 1985) prompted the suggestion that phosphorylation may enhance the space-filling properties of NFs (Carden et al., 1987). This would account for the higher level of NF phosphorylation in the axon as compared to the perikaryon (Sternberger and Sternberger, 1983). A correlation between NF phosphorylation state and axonal caliber has been noted in studies with Trembler mice (de Waegh et al., 1992). The same correlation has been established in cultured DRG neurons. Myelination increases the caliber of axons in these cultures (Windebank et al., 1985), and we have shown that this change is accompanied by an increase in the phosphorylation state of NF-H (Athlan, E., unpublished results). Similarly, axonal narrowing at the node of Ranvier is accompanied by a reduction in NF phosphorylation (Mata et al., 1992).

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