Increased Phosphorylation of the Amino-terminal Domain of the Low Molecular Weight Neurofilament Subunit in Okadaic Acid-treated Neurons*

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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, M. G., Athlani, E. S., and Mushynski, W. E. (1992) Biochem. Biophys. Res. Commun. 186, 524–530). 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 carboxyl-terminal, domain phosphorylation in the low molecular weight (NF-L) subunit in the Triton X-100-soluble and -insoluble fractions. The purified catalytic subunit of protein phosphatase-2A dephosphorylated P-labeled NF-L and the middle molecular weight subunit from okadaic acid-treated cultures, whereas the catalytic subunit of protein phosphatase-1 had no effect. In the case of NF-L, 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.

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 Laske, 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 (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 focused 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-Delahousse 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, kinase, the component required for protein phosphatase-1 (PP-1) activation, was also shown to phosphorylate NF subunits 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 aluminum, 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., 1991). Recent studies show that the PP-1 and protein phosphatase-2A (PP-2A) inhibitory 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 rubra/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, 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; Matsuzaka 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.

**EXPERIMENTAL PROCEDURES**

**Materials**—Okadaic acid and calyculin A were purchased from LC Services (Woburn, MA). Carrier-free 32P, was from ICN Biomedicals (Mississauga, Ontario, Canada).

**SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**—Gel electrophoresis and Western blotting were performed as described previously (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 SCIS camera followed by analysis using the Millipore Bio-Image analyzer. Radioactivity was quantified with the Fuji BAS2000 PhosphoImager.

**Immunoprecipitation**—Cells were harvested in cytoskeleton extraction buffer (CSK buffer) consisting of 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 2 mM EGTA, 2 mM levamisol, 1 mM phenylmethylsulfonyl fluoride and centrifuged for 15 min at 13,000 × 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 Tris-HCl, 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 described previously (Sacher et al., 1992). For metabolic labeling cells were incubated with 0.5 μCi of carrier-free 32P/mI of P3, free medium (Flow Laboratories, McLean, VA) for 3 h prior to OA treatment.

**Phosphatase Treatment of Immunoprecipitated Proteins**—For phosphatase treatment of 32P-labeled immunoprecipitates, samples were incubated for 5 h at 30 °C, prior to elution from the protein A-Sepharose beads (see above), in 65 mM Tris-HCl, pH 7, 1 mM MgCl2, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride x 2 PP-2A, PP-1, (1.5 μg/ml) and OA as described in the figure legends.

**Chemical Cleavage of Polypeptides**—Immunoprecipitated 32P-Labeled NF-L was resolved by SDS-PAGE, located by autoradiography, and excised. The gel slice was rehydrated in N-chlorosuccinimide (NCS) buffer (1 g of urea, 1 ml of water, 1 ml of acetic acid) (Lischwe and Ochs, 1982) and incubated at room temperature for 1.5 h in the same buffer containing 2 μg/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 Du Pont Lightning Plus intensifying screen (Swanson and Shank, 1978) and quantified as described above.

**Preparation of Protein Phosphatase-1 (PP-1) and Phosphatase-2A (PP-2A)—**The catalytic subunits of PP-1 and PP-2A (PP-1, and PP-2A, 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 described previously (Julien and Mushynski, 1982). Unlabeled phosphoamino acid 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 h 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 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, whereas 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 (Inglesby 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 32P-labeled 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 1 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 carboxyl-terminal to amino-terminal 32P phosphorylation (C/N ratios) provides a means for assessing the location of phosphorylation sites on NF-L following OA treatment. DRG cultures were labeled with 32P, followed by a
1-h treatment with 1 μM OA. Samples were fractionated into Triton X-100-soluble and -insoluble fractions, and immunoprecipitated NF-L was treated with NCS. As shown in Fig. 2 the C/N ratio of Triton X-100-soluble and -insoluble NF-L from OA-treated cells was reduced by about 6-fold in comparison with that of the untreated control. It is interesting to note that the C/N ratios of both the Triton X-100-soluble and -insoluble NF-L from OA-treated cultures were the same. These results indicate that although OA treatment causes a preferential increase in amino-terminal domain phosphorylation in NF-L, the Triton X-100-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 X-100-soluble and -insoluble subunits, the Triton X-100-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. 1, A and B), we examined whether PP-2A was capable of acting on NF-M and NF-L from OA-treated cultures. 32P-Labeled 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, or purified PP-1, 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 caused a reduction of about 50% in the amount of 32P in both NF-M and NF-L relative to non-phosphatase-treated subunits from OA-treated cultures, whereas OA, at 10 nM, inhibited this PP-2A-dependent decrease (Fig. 3B, compare lanes 3 and 4). Purified PP-1, had no effect on the 32P 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 could remove the phosphate 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).

![Fig. 2. Gel electrophoretic analysis of the amino- and carboxyl-terminal halves of chemically cleaved NF-L from untreated and okadaic acid-treated DRG cultures. DRG cultures were labeled with 32P, for 3 h and either untreated (lane 1) or treated with 1 μM OA for 1 h (lanes 2 and 3). NF-L was immunoprecipitated from the Triton X-100-insoluble (lane 2) or Triton X-100-soluble (lane 3) fractions. Protein was resolved by SDS-PAGE and digested with NCS (see "Experimental Procedures"). C and N refer to the carboxyl-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 Mf (x 102) standards are shown to the left of the autoradiograph. C/N ratios are shown at the bottom of each lane.](image)

![Fig. 3. Gel electrophoretic analysis of NF subunits from OA-treated DRG cultures after in vitro treatment with PP-1, and PP-2A. 32P-Labeled NF-L and NF-M were immunoprecipitated from control or OA-treated (1 h) DRG cultures (OA). Protein phosphatase treatment (PP-1, and PP-2A) was carried out prior to elution of the subunits from the protein A-Sepharose beads as described under "Experimental Procedures". Addition of OA during phosphatase treatment at either 10 nM or 1 μM is indicated above each lane. Equal amounts of the proteins were fractionated by SDS-PAGE, and the positions of the two subunits are shown to the left of the autoradiograph (A). B shows a histogram obtained by quantifying the autoradiograph from A. Lanes 1–6 in B correspond to the six lanes of the autoradiograph in A. Data pertaining to NF-L are indicated by stippled bars, whereas those for NF-M are represented by solid black bars.](image)
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 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 X-100-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, 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), glial fibrillary acidic protein (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).

OA 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, but not PP-1, 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 mitogen-associated protein 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, 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, of ~50% of the 32P from OA-treated NF-M implies that the enzyme may also dephosphorylate sites in the carboxyl-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 had no effect on the 32P 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-treated subunits which were obtained from cells treated with OA for 1 h (Fig. 4 and Sacher et al., 1992). Alternatively, PP-1 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 X-100-insoluble and -soluble subunits (Fig. 3) and the higher specific radioactivity of the latter as compared with 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 glial fibrillary acidic protein network, found in the Schwann cells of the DRG cultures, which was shown to be complete within 1 h. 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 X-100-soluble NFs in this report is that the keratins were found in a naturally occurring soluble pool made up of tetramers, whereas the OA-induced soluble pool in this study was composed of large oligomers.

2 M. G. Sacher, E. S. Athlan, and W. E. Mushynski, manuscript in preparation.

3 M. G. Sacher, E. S. Athlan, and W. E. Mushynski, unpublished observation.
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 cytoskeleton network observed during Xenopus oocyte maturation may be due to a severing activity, similar to the one seen 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 X-100-soluble, further studies are required to define the role of domain-specific phosphorylation in NF dynamics.

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REFERENCES


Takahashi, T., and DeLorenzo, R. J. (1992) Trends Neurosci. 15, 70–75