Identification of Serine 473¹ as a Major Phosphorylation Site in the Neurofilament Polypeptide NF-L

Zuo-Shang Xu, Wu-Schyong Liu, and Mark Willard

Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110

Neurofilaments are composed of 3 polypeptides designated NF-H, NF-M, and NF-L, all of which are subject to posttranslational phosphorylation. It has been suggested that phosphorylation of the NF-L polypeptide can influence the assembly of NF-L into filaments, but the sites at which NF-L is phosphorylated are unknown. To locate these phosphorylation sites, we have identified phosphopeptides of NF-L by labeling them with ³²P both *in vitro* and in cultured neurons and also by observing their change in chromatographic behavior after they have been treated with phosphatase. We report here that serine 473, in the carboxy-terminal tail domain of NF-L, is a major substrate *in vitro* for protein kinases endogenous to a crude cytoskeleton-containing fraction. Moreover, serine 473 is a major phosphorylation site *in vivo*; in neurofilaments isolated from rat spinal cord, approximately 73% of serine 473 was phosphorylated, and accounted for at least one-third of the total phosphate associated with NF-L. The identification of this phosphorylation site in NF-L provides a criterion for identifying the protein kinase that phosphorylates NF-L and raises the question of its function.

Neurofilaments, 10-nm-diameter intermediate filaments that are a major cytoskeletal element of many axons, comprise 3 polypeptides designated NF-H (*MW* ≈ 110K), NF-M (*MW* ≈ 100K), and NF-L (*MW* ≈ 61K). Each of these polypeptides has structural features characteristic of the family of intermediate filament proteins: (1) a central α -helical rod domain that interacts with other rod domains to form tetrameric subunits, (2) an N-terminal head domain that is important for polymerization, and (3) a C-terminal tail region of variable length that is thought to extend from the filament and interact with the surrounding environment (Fig. 1; for reviews, see Steinert and Roop, 1988; Inagaki et al., 1989). After they are synthesized in the cell bodies of neurons, all 3 neurofilament polypeptides are phosphorylated, but to different extents: 3, 6, and 13 mol phosphate/mol polypeptide for NF-L, NF-M, and NF-H, respectively in rat (Julien

and Mushynski, 1982; the reported values have been corrected to reflect more recent determinations of the molecular weights of the subunits). Phosphorylation sites on NF-H and NF-M are located in the middle portion of the C-terminal tail domain (Julien and Mushynski, 1983; Geisler et al., 1985b), and the amino acid sequences of several of these sites have been identified (Geisler et al., 1987). Although 3 phosphorylated chymotryptic fragments of mouse NF-L have been isolated (Sihag and Nixon, 1989), the specific sites at which NF-L is phosphorylated have not been identified previously.

All 3 neurofilament polypeptides can be phosphorylated *in vitro* by protein kinase(s) that are components of crude cytoskeleton subcellular fractions (Shecket and Lasek, 1982; Julien et al., 1983). Whether these protein kinases phosphorylate the same sites that are phosphorylated *in vivo* has not been determined previously. The phosphorylation sites on neurofilament proteins are potentially important in regulating cytoskeletal structure (Evans and Fink, 1982; Celis et al., 1983; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985; Westwood et al., 1985; Burke and Gerace, 1986; Inagaki et al., 1987; Geisler and Weber, 1988; Glicksman et al., 1987; Angelides et al., 1989). Here we describe the identification of a major phosphorylation site, serine-473, on neurofilament peptide NF-L from rats; this site is phosphorylated both in the living animals and *in vitro* by protein kinases in crude preparations of cytoskeleton.

Materials and Methods

Preparation of neurofilament proteins. A Triton X-100-insoluble pellet was produced by homogenization of frozen rat spinal cord in a buffer containing 50 mM phosphate (pH 7.0), 1 mM dithiothreitol (DTT), 1 mM EDTA, 150 mM NaCl, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1% Triton-X100, followed by centrifugation at 13,000 × *g* for 30 min. The pellet was washed 3 times by resuspension in the same buffer containing 0.9 M sucrose followed by centrifugation. The final pellet was homogenized in 1 ml/gm of original tissue of phosphorylation buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM DTT, 1 mM EDTA, 5 mM MgSO₄, 100 μ M ³²P-ATP, 5 Ci/mmol) without ATP and stored at –70°C. A procedure developed by Dan Soppet (D. Soppet, L. Beasley, and M. Willard, unpublished observations) was used to purify NF-L. The Triton X-100-insoluble pellet was resuspended in Buffer A (20 mM Na₂HPO₄, 7 M urea, pH 7.5) and, after clarification by centrifugation in a microcentrifuge for 15 min, was chromatographed on an HPLC-DEAE column (Waters, Protein Pak, SPW) that was developed according to Program I (Table 1). NF-L was further purified by reverse-phase HPLC chromatography on a C4 column (Vydac, 214 TP54) developed according to Program II (Table 1). The fractions containing NF-L were dried under vacuum and either stored at –70°C or digested with trypsin.

Labeling neurofilaments *in vitro*. The neurofilament-rich Triton-X-100-insoluble pellet was prepared from rat spinal cord as described above and resuspended by homogenization in phosphorylation buffer. The mixture was incubated at 37°C for 1 hr and centrifuged at 12,000 × *g* at 4°C for 10 min. The pellet was then dissolved in Buffer A, and

Received Oct. 30, 1989; revised Dec. 14, 1989; accepted Dec. 18, 1989.

We thank Janis Branneky and Sue Eads for typing; D. Soppet, L. Beasley, A. Loewy, R. Cheney, Dr. S. Schuh, and S. Spencer for their advice and assistance with this work, which was supported by grants from the National Institutes of Health (EY06082, NS22828).

Correspondence should be addressed to Mark Willard, Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110.

¹ Although NF-L from rat was used in these experiments, serine 473 refers to the numbering scheme of Lewis and Cowan (1986) for mouse NF-L, because the sequence of rat NF-L has not been published. Serine 473 corresponds to serine 471 in the numbering scheme of Julien et al. (1986) for mouse NF-L.

Copyright © 1990 Society for Neuroscience 0270-6474/90/061838-09\$02.00/0

Figure 1. Schematic diagram of NF-L, based on the amino acid sequence from mouse (Lewis and Cowan, 1986). The α -helical regions and nonhelical regions are designated by open and hatched bars, respectively. The location of the amino terminal sequences of the tryptic phosphopeptides I–IV within the amino acid sequence of the tail domain are indicated by arrows. The decapeptide C (filled bar) is a product of digestion of peptide IV with V8 protease and contains serine 473, which we demonstrate here is a major site of phosphorylation. The dots indicate amino acids, which, based upon our sequencing results, may be different in rat, i.e., A, S, and an amino acid of uncertain identity, respectively. Serine 473 corresponds to serine 471 in the sequence of mouse NF-L reported by Julien et al. (1986). The sequence of rat NF-L has not been published.

NF-L was prepared by HPLC-DEAE and reverse-phase chromatography as described above.

SDS-PAGE of proteins. Proteins were separated on SDS-polyacrylamide gels containing gradients of 4–12% acrylamide, as described previously (Glicksman et al., 1987). The gels were either exposed to X-ray film or stained and destained as described by Fairbanks et al. (1971).

Tryptic digestion of NF-L. Two methods were used: the first was a procedure modified from Yu and Czech (1984) and was used for digestion of NF-L labeled with ^{32}P , either *in vitro* or in dorsal root ganglion cell cultures. The proteins of the Triton-X-100-insoluble pellet were first fractionated by HPLC-DEAE chromatography as described above. The fractions containing NF-M and NF-L were then desalted on an HPLC gel-filtration column (Bio-Gel TSK 40 column, 300 $\times$ 7.5 mm, from Bio-Rad) using 10 mM NH_4HCO_3 as the eluting buffer. The proteins were dried under vacuum, redissolved in gel loading buffer (10 mM Tris, pH 8.0, 5 mM EDTA, 4 mM DTT, 2% SDS, 30% glycerol, and a trace of bromophenol blue) and electrophoresed on a gradient SDS-polyacrylamide gel. The gel (undried) was exposed to X-ray film to localize the NF-L band, which was excised and chopped into small pieces that were then incubated with 3 ml of a solution containing trypsin (0.1 mg/ml) and NH_4HCO_3 (50 mM). After digestion for 24 hr at 37°C, the supernatant was collected and the gel pieces were washed with 50 mM NH_4HCO_3 (3 ml) for 5 hr. Both supernatants were lyophilized.

The second method, used to digest NF-L that had been purified by HPLC-DEAE and C4 chromatography as described above, was as follows: Lyophilized NF-L was resuspended in 50 mM NH_4HCO_3 and digested with trypsin at an enzyme to protein ratio of 1:50 for 5 hr at 37°C. The resulting digest was lyophilized.

Mapping and purification of the phosphopeptides. The lyophilized tryptic peptides were dissolved in a solution of 10 mM phosphate at pH 6.9 and chromatographed on a reverse phase C-18 column (Vydac, 218 TP54) on HPLC. Peptides were eluted with a phosphate (pH 6.9) buffered acetonitrile gradient (Program III, Table I). Fractions (one minute) were collected and counted by Cherenkov counting in a Beckman LS-6000 scintillation counter.

For further purification, the fractions containing radioactive phosphopeptides were loaded on a second C-18 column and eluted with an acetonitrile gradient (Program IV, Table I) buffered with TFA (pH 2.0). The radioactive peptides were localized by Cherenkov counting and saved for sequencing.

***S. aureus* V8 protease digestion of the phosphopeptide.** Tryptic phosphopeptide IV (Fig. 1) was purified as described above, dried, and resuspended in a solution of 50 mM citric acid (pH 4.0) and 0.5 mM EDTA. *S. aureus* V8 protease was added to a concentration of 0.1 mg/ml. After the peptide was digested overnight at 37°C, another aliquot of enzyme was added and the digestion was continued for 24 hr. The digest was then fractionated on a C-18 column with an acetonitrile gradient buffered by 0.1% TFA (Program V, Table I).

Dephosphorylation of phosphopeptides. Two types of experiments were carried out. First, decapeptide C (Fig. 1) containing serine 473 was dissolved in 100 μl of a solution containing NH_4HCO_3 , 1 mM ZnCl_2 , 1 mM MgCl_2 , 150 mM KCl and incubated with 0.7 units of *E. coli* alkaline phosphatase (Sigma, Type III-N) overnight at 37°C. Second, a mixture of peptides (100 μl), generated from tryptic peptide IV (Fig. 1) by *S. aureus* V8 protease was treated with 1 mM PMSF to inactivate the protease. Potato acid phosphatase (10 μl ; Sigma, Type III) was sedimented by centrifugation at 4°C and the peptide mixture (in a solution of 50 mM citric acid, pH 4.0) was added to the pellet. An equal volume of 50 mM citric acid (pH 5.6) was added to adjust the pH to 4.8, and MgSO_4 was added to achieve a concentration of 1 mM. The mixture was incubated at room temperature overnight and then analyzed directly on a C-18 column using Program V, Table I.

Microsequencing and modification of peptides. The amino acid sequencing was performed on a gas-phase sequencer (Applied Biosystems) as described by Hewick et al. (1981). All chemicals were purchased from Applied Biosystems, Inc. The standard degradation run (03RPTH) was used. The presence of phosphoserine in the peptide was confirmed by the method of Meyer et al. (1986). The method of Merrifield (Stewart and Young, 1984) was used to synthesize peptide C (Fig. 1) on an Applied Protein Technologies (Cambridge, MA) PSS-80 synthesizer.

Table 1. HPLC gradients^a

Program #	Time (min)	Buffer A	Buffer B	Flow rate (ml/min)
I		20 mM PO ₄ (pH 7.5)	0.45 M PO ₄ (pH 7.0)	0.5
		7 M urea	7 M urea	
	0	90%	10%	
	15	90%	10%	
II	50	0%	100%	1.5
		0.1% TFA	0.1% TFA	
			95% acetonitrile	
	0	60%	40%	
III	5	60%	40%	1.0
	30	45%	55%	
		10 mM PO ₄ (pH 6.9)	5 mM PO ₄ (pH 6.9)	
			70% acetonitrile	
IV	0	100%	0%	1.0
	20	94%	6%	
	60	77%	23%	
	90	62%	38%	
	110	48%	52%	
		0.1% TFA	0.1% TFA	
V			75% CH ₃ CN	1.0
	0	100%	0%	
	60	40%	60%	
		0.1% TFA	0.1% TFA	
V			75% acetonitrile	1.0
	0	100%	0%	
	80	60%	40%	

^a Note: All changes in buffer were linear with time.

Protein assay. The bicinchoninic acid (PCA, Pierce Chemical Company) assay for proteins was used as described by Smith et al. (1985).

Phosphate assay. The amount of phosphate bound covalently to NF-L was estimated by the method of Buss and Stull (1983) with slight modifications. NF-L (50 µg) was dissolved in 10 µl of a solution containing 20 mM NaHCO₃ (pH 8.2), 8 M urea. H₂O (90 µl, 4°C) was then added to dilute the urea to 0.8 M. Trichloroacetic acid (TCA; 20% wt/vol, 100 µl) was added and the protein precipitate was collected by

centrifugation. The protein pellet was then dissolved in 20 µl of SDS (1%) and diluted by addition of 80 µl H₂O. The TCA precipitation was repeated, and the pellet was heated to 90°C in 200 µl of 16% TCA for 20 min. After centrifugation, the pellet was dissolved (in less than 1 min) in 100 µl ice-chilled 0.1 N NaOH, and the protein was precipitated by addition of 100 µl of 20% TCA. The final pellet was dissolved in 50 µl of 1% SDS. A sample (10 µl) was used to measure the protein concentration as described above. The remainder was lyophilized, and phosphate was measured as described by Buss and Stull (1983).

Labeling neurofilaments in rat dorsal root ganglion culture. Dorsal root ganglia were dissected from 1-d-old rat pups and dissociated by incubation and trituration 2 times (10 min each) with 0.1% trypsin and collagenase in Puck's saline without potassium and magnesium. The cells were then plated on poly-L-lysine-coated tissue culture plates (Falcon) and grown for 1 week in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (Gibco). To label the neurofilaments, the medium was replaced with a medium containing ³²P-ATP (2 mCi, 233 Ci/mg, New England Nuclear, NEX-053), and 5% fetal calf serum in DMEM lacking phosphate. The cultures were labeled for 48 hr. The radioactive media was then removed, and the neurons were scraped from the dishes with a rubber policeman. Approximately 2 mg protein from a Triton-X-100 insoluble fraction of rat spinal cord, prepared as described above, was added as carrier, and a Triton-X-100-insoluble pellet from the cultured cells was then prepared, as described above.

Results

Identification of serine 473 as a major site for phosphorylation of NF-L by cytoskeleton-associated protein kinases in vitro Neurofilaments can be phosphorylated with ³²P-ATP by an endogenous protein kinase system in a cytoskeletal subcellular fraction (insoluble in nonionic detergent) from spinal cord (Shekhet and Lasek, 1982; Julien et al., 1983). Figure 2 shows the time course of incorporation of ³²P into the neurofilament polypeptides in such a preparation. After 1 hr of incubation,

Figure 2. Time course of incorporation of ³²P into the neurofilament proteins *in vitro*. A crude cytoskeleton subcellular fraction from rat spinal cord was incubated with ³²P-ATP as described in Materials and Methods. At the indicated times, equal amounts of neurofilament proteins were separated by SDS-PAGE, and radioactivity was determined in bands excised from the gel.

Figure 3. Tryptic peptide map of NF-L phosphorylated *in vitro* with ^{32}P . The tryptic peptides were separated by reverse-phase HPLC on a C-18 column. The dashed line indicates the acetonitrile gradient (Program III, Table 1).

approximately 0.07 mol phosphate was incorporated per mole of NF-L. Phosphorylation was not inhibited by heparin (10 $\mu\text{g}/\text{ml}$, 10 min incubation), which completely inhibits casein kinase II (Hathaway et al., 1980).

To determine the sites in NF-L that are phosphorylated, we purified the labeled NF-L from such a reaction mixture, digested it with trypsin, and separated the resulting peptides by reverse-phase HPLC (Fig. 3). The major radioactive peaks designated I, II, III, and IV corresponded to major peaks that can be generated in living cells as discussed below (see Fig. 7). We further purified the major radioactive peptides in these peaks by reverse-phase chromatography in a buffer of lower pH (see Materials and Methods) and determined the partial N-terminal amino acid sequence of each. A comparison with the complete amino acid sequence of mouse NF-L revealed that all 4 peptides were derived from the C-terminal 20% of the NF-L polypeptide (Fig. 1). Peak I gave rise to 2 overlapping sequences, the major sequence beginning at amino acid 464 and a minor sequence beginning at amino acid 469. Peak II generated these same 2 N-terminal sequences, suggesting that the peptides of peak II differ from those of peak I in their C-terminal extent. Peaks III and IV each generated a single N-terminal sequence beginning at amino acid 439, suggesting that they contain peptides that differ from each other only in their C-terminal extents.

The peptide of peak IV is a partial digestion product that includes part or all of the peptides of peaks I and II. This is

indicated by the observations that either higher trypsin concentrations or extended proteolysis reduced the yield of peaks III and IV, while the yield of peaks I and II increased, and that the sequence of peptide IV extends for an undetermined distance into the sequence of peptides I and II (Fig. 1). Because peak IV contains a single sequence that may potentially include the sequences of all 4 peaks, and because it could be generated with the highest yield, we further investigated the sites of phosphorylation of this peptide.

Peptide IV was digested at a low pH (see Materials and Methods) with *S. aureus* V8 protease, which cleaves predominantly at glutamic acid residues, and the resulting peptides were separated by reverse-phase chromatography (Fig. 4). The peptides in fractions that contained the five major peaks of radioactivity were sequenced completely. Peaks C, D, and E each contain a single sequencable peptide beginning at amino acid 467, and extending for 10, 13, and 14 residues, respectively (Fig. 1). Peaks A and B gave rise to multiple short sequences whose location within the polypeptide could not be assigned unequivocally. We conclude that serine 473, contained in peptides C, D, and E is a major phosphorylation site, for the following reasons. First, serine 473 is the only potential phosphorylation site in peptides C, D, and E. Second, during the sequential Edman degradation used to determine the sequence, serine 473 was recovered exclusively as the dithiothreitol adduct of PTH serine, which indicates that this serine is modified; under these sequencing con-

Figure 4. Separation of peptides derived from tryptic peptide IV (Fig. 3) by digestion with *S. aureus* V8 protease. The five peaks (A–E) were separated by reverse phase HPLC on a C-18 column. The dashed line indicates the acetonitrile gradient (Program V, Table 1).

ditions, unmodified serine is only partially converted to this adduct. These observations indicate that serine 473 is a major site of phosphorylation of NF-L by protein kinases in a cytoskeletal subcellular fraction *in vitro*. They raise the question of whether this site is also phosphorylated in living animals.

Serine 473 of NF-L is highly phosphorylated in living rats

NF-L was purified from rat spinal cord, and peptide C, generated by digestion with trypsin and *S. aureus* V8 protease as described above, was isolated by reverse-phase HPLC. Four observations demonstrate that serine 473, the only potential phosphate acceptor in peptide C, is phosphorylated. First, peptide C was eluted from a C18 reverse-phase HPLC column 2 min earlier than a synthetic decapeptide of the same amino acid sequence, indicating that peptide C contains modified amino acids (Fig. 5 A, C). Second, after we incubated peptide C with *E. coli* alkaline phosphatase, its elution from this column coincided with the elution of the synthetic peptide (Fig. 5 B, C). This shift did not occur when the incubation was performed in the presence of phosphate to inhibit the phosphatase. This indicates that peptide C is phosphorylated. Third, when peptide C was sequenced by sequential Edman degradation, serine 473 was recovered exclusively as the DTT-adduct of PTH-serine, whereas 25% of serine 473 was recovered as PTH serine when either phosphatase-treated peptide C or synthetic peptide C was sequenced. This indicates that serine 473 is modified.

Fourth, to confirm further that serine 473 is phosphorylated, we used the observation that O-modified serine, but not serine, reacts with ethanethiol at high pH, forming S-ethyl cysteine (Meyer et al., 1986). After incubation with ethanethiol, peptide C was

eluted from the reverse-phase columns in 2 peaks that were delayed by 15 and 18 min compared with untreated peptide C (Fig. 6). Sequencing confirmed that both of these peaks contained the decapeptide C with serine 473 converted to S-ethyl cysteine. (One explanation for the difference between these peaks is that they may contain different isomers of the modified peptide.) On the other hand, when phosphatase-treated peptide C or synthetic peptide C was incubated with ethanethiol, their elution from the reverse-phase column was not altered, and sequencing confirmed that serine 473 was not modified. These observations show that serine 473 from rat spinal cord is modified by phosphorylation. By comparing the areas of the peaks corresponding to the phosphorylated and unphosphorylated forms of peptide C in the *S. aureus* V8 protease map of the C-terminal region of NF-L, we estimate that $73 \pm 2\%$ ($n = 3$) of serine 473 is phosphorylated in NF-L isolated from rat spinal cord. (We consider this a minimum estimate, because we do not know how much phosphate is lost from this site during purification of NF-L.) Thus, serine 473 is a major phosphorylation site *in vivo* as well as *in vitro*.

Phosphate content of rat spinal cord NF-L

To determine the total amount of phosphate associated with NF-L in rat spinal cord, we purified NF-L and used the method of Buss and Stull (1983) to estimate that there are 1.3 ± 0.22 ($n = 3$) mol phosphate/mol NF-L. This method employs a series of TCA precipitations to remove phosphate that is not covalently bound to protein. Using ^{32}P -orthophosphate as a tracer, we determined that these precipitations reduce the amount of phosphate contributed by the buffer (Buffer B of Program I in Table 1) to less than 0.01 nmol in the assay sample, which is one-tenth the minimum that can be detected by this assay. However, when we subjected NF-L to a second series of TCA precipitations, in 2 experiments the amount of phosphate measured was 40% lower than after the first series of precipitations. We cannot unequivocally rule out the possibility that this reduction represents the removal of an especially persistent contaminant, in which case a better estimate would be 0.78 mol phosphate/mol NF-L in spinal cord. However, it is more likely that this reduction represents removal of phosphate covalently bound to the protein during the cycles of TCA precipitation. Assuming that a similar fraction of covalently bound phosphate was removed during the first sequence of TCA precipitations, our best estimate for the average phosphate content of NF-L is 2.2 mol phosphate/mol NF-L. In this case, serine 473 would account for approximately 30% of the total phosphate in NF-L.

Phosphatase sensitivity of peptides

In an effort to identify additional phosphorylation sites in the C-terminal region of NF-L, we digested the peptide from peak IV (Fig. 3) with *S. aureus* V8 protease, and, after inhibiting the *S. aureus* V8 protease with PMSF, incubated the resulting peptides with potato acid phosphatase (see Materials and Methods). The resulting peptides were separated by C-18 reverse-phase HPLC, and their elution was compared with control peptides that had been prepared identically, except that during the incubation with phosphatase, 50 mM phosphate had been included to inhibit the phosphatase. Although peptide C, containing phosphorylated serine 473, was partially converted to its unphosphorylated form, indicating that dephosphorylation had occurred, no other peaks were altered in a manner suggesting that they were dephosphorylated. However, this procedure would

Figure 5. Change of migration of the decapeptide C (Fig. 4) on C-18 reverse-phase HPLC after treatment with *E. coli* alkaline phosphatase. After treatment with phosphatase (B) peptide C was eluted later than a control sample (A) that was identical except that the phosphatase had been inhibited by 50mM phosphate. The phosphatase-treated peptide (B) coeluted with a synthetic decapeptide with the same sequence as peptide C (C). The dashed line indicates the acetonitrile gradient (Program V, Table 1).

not have detected additional sites if (1) they were resistant to our phosphatase treatment, (2) only a small fraction of the site is phosphorylated, as would be the case if the additional phosphate was distributed among a large number of sites, (3) they are contained in peptides that are too short to adsorb to the reverse-phase column, or to be detected by ultraviolet absorbance, and (4) the sites were located between the C-terminal end of peptide IV and the C-terminal end of NF-L.

Phosphorylation of NF-L in cultured dorsal root ganglia

To examine the possibility that other phosphorylation sites reside outside the C-terminal tail region represented by tryptic peptides I–IV (Figs. 1, 3), we incubated cultures of dorsal root ganglia from 1-d-old rats with ^{32}P -orthophosphate for 48 hr, isolated NF-L, digested it with trypsin, and separated the re-

Figure 6. Change of migration of decapeptide C on C-18 reverse-phase HPLC after modification by ethanthal. After treatment with ethanthal, peptide C generated 2 peaks (A) that were eluted later than untreated peptide C (Fig. 5A) or peptide C that had been treated with ethanthal after incubation with *E. coli* alkaline phosphatase (B), or a synthetic decapeptide with the same sequence as peptide C treated with ethanthal (C). This confirms that peptide C is modified by phosphorylation. Chromatography conditions were the same as in Figure 4.

sulting peptides by reverse-phase chromatography. Figure 7 illustrates that the major radioactive peaks were eluted at times corresponding to peaks I–IV that can be generated by phosphorylation *in vitro* as described above (Fig. 3). On the other hand, none of the other peaks which had been generated by phosphorylation *in vitro* were generated by the labeling of these living cells. Thus, all of the labeling that we observed in these cultured cells is associated with peaks that, when labeled *in vitro*, contain only peptides from the C-terminal 20% of NF-L. (However, we would not have detected a phosphopeptide from the head region that co-chromatographed with these C-terminal peptides if its amino terminus was blocked.) The amount of labeled peptide that was generated by these dorsal root ganglion cell cultures was insufficient to confirm the sequence of these peptides and the sites that were phosphorylated within them.

Discussion

The foregoing evidence shows that serine 473, located in the carboxyterminal tail domain, is a major phosphorylation site in NF-L. Approximately 73% of serine 473 in NF-L isolated

Figure 7. Tryptic phosphopeptide map of NF-L labeled with ^{32}P in living dorsal root ganglion cells. The major labeled peptides, designated I–IV, comigrate with the tryptic peptides of the same designation generated by *in vitro* phosphorylation of purified H (Fig. 3). Method 1 (Materials and Methods) was used to generate the peptide map. The dashed line indicates the acetonitrile gradient (Program I, Table 1). The conditions of chromatography were identical to those of Figure 3.

from rat spinal cord is phosphorylated, accounting for at least one-third of the phosphate associated with NF-L. This site is also a major substrate for protein kinases endogenous to a crude, cytoskeleton-containing subcellular fraction.

The sequence surrounding serine 473, EPPSEGEAE, is represented in the sequence of all of the mammalian NF-L polypeptides that have been sequenced, i.e., mouse (Julien et al., 1986; Lewis and Cowan, 1986), pig (Geisler et al., 1985a), human (Julien et al., 1987), and rabbit (Soppet et al., unpublished observations), as would be anticipated if elements of this sequence serve as the recognition site for a functionally important protein kinase. This sequence is characteristic of those recognized by casein kinase II (Marin et al., 1986); i.e., acidic residues are located 3 and 5 amino acids away from the substrate serine on the C-terminal side. However, serine 473 can apparently be phosphorylated by protein kinases other than casein kinase II, because we observed that phosphorylation of NF-L *in vitro* by protein kinases endogenous to a cytoskeletal fraction was not inhibited by heparin at a concentration (10 $\mu\text{g}/\text{ml}$) that inhibits casein kinase II completely (Hathaway et al., 1980). Protein kinases in similar cytoskeletal subcellular fractions (Runge et al., 1981; Sheckel and Lasek, 1982; Julien et al., 1983; Toral-Delbauffe et al., 1986; Wible et al., 1989) include (1) a calcium-cyclic nucleotide-independent kinase that is inhibited by high concentrations of heparin (100 μM) and is apparently responsible for the phosphorylation of a C-terminal chymotryptic fragment (L1) of NF-L *in vitro* (Sihag and Nixon, 1989); (2) a calcium-calmodulin-stimulated protein kinase activity (Vallano et al., 1985); and (3) a cAMP-dependent protein kinase associated with microtubules that phosphorylates NF-M preferentially (Leterrier et al., 1981). The identification of serine 473 as a specific site of phosphorylation of NF-L should facilitate the definitive identification of the protein kinase responsible for its phosphorylation.

Serine 473 of NF-L resembles the identified multiple phosphorylation sites in NF-H and NF-M (KSP, PVSF, and AESP; Geisler et al., 1987) with respect to its location in an acidic

region in the middle of the C-terminal tail of the polypeptide, and with respect to its proximity to proline. However, the predominant phosphorylation sites in NF-H (KSP) differ substantially from serine 473 of NF-L in that the proline is on the C-terminal side of the phosphorylated serine, which is adjacent to a positively charged amino acid.

Several potential functions for the phosphorylation of serine 473 in the C-terminal tail region of NF-L are worthy of consideration. First, the comparable C-terminal tail of NF-H can project from the filament and interact with neighboring organelles (Hirokawa et al., 1984); it has been considered that these projections might be regulated by phosphorylation of the tail region (e.g., Glicksman et al., 1987). By analogy, the phosphorylation of serine 473 might regulate the projection and interactions of the C-terminal tail of NF-L. Second, neurofilament polypeptides within axons appear to comprise both a stationary phase (accounting for more than half of the neurofilament polypeptides in the mouse optic nerve) and a moving phase, presumed to maintain the stationary phase by providing newly synthesized proteins from the cell body (Nixon and Logvinenko, 1986). The phosphorylation of serine 473 (of which approximately 70% is phosphorylated in rat spinal cord) might be associated with the transition of NF-L between the moving and stationary phases, or with functions specific to one of these phases.

A third functional possibility is raised by evidence that phosphorylation of other intermediate filament proteins regulates their polymerization and organization within the cell. Vimentin, keratin, and the nuclear lamins undergo increased phosphorylation that may be associated with depolymerization or reorganization of the cytoskeleton prior to cell division (Evans and Fink, 1982; Celis et al., 1983; Ottaviano and Gerace, 1985; Westwood et al., 1985). Moreover, the phosphorylation of desmin and vimentin (by cAMP-dependent kinase and/or protein kinase C) and the lamins inhibits filament formation *in vitro* (Miake-Lye and Kirschner, 1985; Burke and Gerace, 1986; Inagaki et al., 1987; Geisler and Weber, 1988). It has been stated that the phosphorylation state of NF-L also influences its polymerization *in vitro* (Angelides et al., 1989). However, unlike serine 473 of NF-L, all of the identified phosphorylation sites of these protein kinases in vimentin and desmin are in the amino terminal head domain (Geisler and Weber, 1988; Ando et al., 1989), a region that, in the case of desmin, keratin, and vimentin, has been shown to be critical for polymerization (Traub and Vargias, 1983; Kaufman et al., 1985; Albers and Fuchs, 1987). In contrast, the C-terminal region of keratin and desmin, which is comparable to the region of NF-L that contains serine 473, is not essential for polymerization (Kaufman et al., 1985; Albers and Fuchs, 1987). Thus, although we cannot rule out the possibility that phosphorylation of serine 473 regulates polymerization of NF-L, its location is not that which would be expected for such a function.

This consideration raises the question of whether NF-L contains additional phosphorylation sites in the head region that could regulate assembly; alternatively, if such sites were only involved in rapid rearrangements of the cytoskeleton associated with cell division, they might be irrelevant to neurofilaments, which typically are first expressed at approximately the time of the final cell division (Tapscott et al., 1981a, b; Shaw and Weber, 1983). Our failure to identify such sites in NF-L does not directly address this question, because we analyzed only NF-L associated with polymerized filaments, and would not have detected sites

that are occupied only in depolymerized subunits. Furthermore, our chemical determination that there are 2.2 mol phosphate/mol NF-L, which falls within the range of previous estimates (Jones and Williams, 1982; Julien and Mushinski, 1982; Wong et al., 1984), suggests that there are at least 2 phosphorylation sites in addition to serine 473. Although all of the ^{32}P incorporated into NF-L in dorsal root ganglion cell cultures was associated with peptides that cochromatographed with peptides from the C-terminal 20% of NF-L, an examination of the phosphatase sensitivity of subfragments of this region (derived from rat spinal cord) served to identify only the phosphopeptide-containing serine 473, leaving the question of the location of the additional site(s) unanswered. It is relevant to this question that Sihag and Nixon (1989) observed that 3 chymotryptic fragments of mouse L (L1–L3) were labeled with ^{32}P -orthophosphate *in vivo*. L1 was apparently derived from the C-terminal portion of NF-L and presumably reflects the phosphorylation of serine 473. L2 was a substrate for cAMP-dependent protein kinase *in vitro*, suggesting, by analogy to other intermediate filaments, that it may be derived from the head region. Identification of the sites that are phosphorylated in peptides L1 and L2 may serve to account for the remainder of the phosphate associated with NF-L.

References

- Albers K, Fuchs E (1987) The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. *J Cell Biol* 105:791–806.
- Ando S, Tanabe K, Gonda Y, Sato C, Inagaki M (1989) Domain- and sequence-specific phosphorylation of vimentin induces disassembly of the filament structure. *Biochemistry* 28:2974–2979.
- Angelides KJ, Smith KE, Takeda M (1989) Assembly and exchange of intermediate filament proteins of neurons: neurofilaments are dynamic structures. *J Cell Biol* 108:1495–1506.
- Burke B, Gerace L (1986) A cell-free system to study reassembly of the nuclear envelope at the end of mitosis. *Cell* 44:639–652.
- Buss JE, Stull JT (1983) Measurement of chemical phosphate in proteins. *Methods Enzymol* 99:7–14.
- Celis JE, Larsen PM, Fey SJ, Celis A (1983) Phosphorylation of keratin and vimentin polypeptides in normal and transformed mitotic human epithelial amnion cells: behavior of keratin and vimentin filaments during mitosis. *J Cell Biol* 97:1429–1434.
- Evans RM, Fink LM (1982) An alteration in the phosphorylation of vimentin-type intermediate filaments is associated with mitosis in cultured mammalian cells. *Cell* 29:43–52.
- Fairbanks G, Steck TL, Wallach DFH (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606–2617.
- Geisler N, Weber K (1988) Phosphorylation of desmin *in vitro* inhibits formation of intermediate filaments; identification of three kinase A sites in the aminoterminal head domain. *EMBO J* 7:15–20.
- Geisler N, Plessmann U, Weber K (1985a) The complete amino acid sequence of the major mammalian neurofilament NF-L. *FEBS Lett* 182:475–478.
- Geisler N, Fischer S, Vandekerckhove J, Damme JV, Plessmann U, Weber K (1985b) Protein-chemical characterization of NF-H, the largest mammalian neurofilament component; intermediate filament-type sequences followed by a unique carboxy-terminal extension. *EMBO J* 4:57–63.
- Geisler NG, Vandekerckhove J, Weber K (1987) Location and sequence characterization of the major phosphorylation sites of the high molecular mass neurofilament proteins M and H. *FEBS Lett* 221:403–407.
- Glicksman MA, Soppet D, Willard MB (1987) Posttranslational modification of neurofilament polypeptides in rabbit retina. *J Neurobiol* 18:167–196.
- Hathaway GM, Lubben TH, Traugh JA (1980) Inhibition of casein kinase II by heparin. *J Biol Chem* 255:8038–8041.
- Hewick RM, Hunkapillar MW, Hood LE, Dryer WJ (1981) A gas-liquid solid phase peptide and protein sequencer. *J Biol Chem* 256:7990–7997.
- Hirokawa N, Glicksman MA, Willard MB (1984) Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. *J Cell Biol* 98:1523–1536.
- Inagaki M, Nishi Y, Nishizawa K, Matsuyama M, Sato C (1987) Site-specific phosphorylation induces disassembly of vimentin filaments *in vitro*. *Nature* 328:649–652.
- Inagaki M, Gonda Y, Ando S, Kitamura S, Nishi Y, Sato C (1989) Regulation of assembly-disassembly of intermediate filaments *in vitro*. *Cell Struct Funct* 14:279–286.
- Jones SM, Williams RC Jr (1982) Phosphate content of mammalian neurofilaments. *J Biol Chem* 257:9902–9905.
- Julien J-P, Mushynski WE (1982) Multiple phosphorylation sites in mammalian neurofilament polypeptides. *J Biol Chem* 257:10467–10470.
- Julien J-P, Mushynski WE (1983) The distribution of phosphorylation sites among identified proteolytic fragments of mammalian neurofilaments. *J Biol Chem* 258:4019–4025.
- Julien J-P, Smoluk GD, Mushynski WE (1983) Characteristics of the protein kinase associated with rat neurofilament preparations. *Biochim Biophys Acta* 755:25–31.
- Julien J-P, Meyer D, Flarell D, Hurst J, Grosveld F (1986) Cloning and developmental expression of the murine neurofilament gene family. *Mol Brain Res* 1:243–250.
- Julien J-P, Grosveld F, Yazdankbakhsh K, Flarell D, Meijer D, Mushynski W (1987) The structure of a human neurofilament gene (NF-L): a unique exon-intron organization in the intermediate filament gene family. *Biochim Biophys Acta* 909:10–20.
- Kaufmann E, Weber K, Geisler N (1985) Intermediate filament forming ability of desmin derivatives lacking either the amino-terminal 67 or the carboxy-terminal 27 residues. *J Mol Biol* 185:733–742.
- Leterrier J-F, Liem RKH, Shelanski ML (1981) Preferential phosphorylation of the 150,000 molecular weight component of neurofilaments by a cyclic AMP-dependent microtubule-associated protein kinase. *J Cell Biol* 90:755–760.
- Lewis SA, Cowan NJ (1986) Anomalous placement of introns in a member of the intermediate filament multigene family: an evolutionary conundrum. *Mol Cell Biol* 6:1529–1534.
- Marin O, Meggio F, Marchiori F, Borin G, Pinna LA (1986) Site specificity of casein kinase-II from rat liver cytosol. *Eur J Biochem* 160:239–244.
- Meyer HE, Hoffman-Posorske E, Korte H, Heilmeyer LMG, Jr (1986) Sequence analysis of phosphoserine-containing peptides, modification for picomolar sensitivity. *FEBS Lett* 204:61–66.
- Miake-Lye R, Kirschner MW (1985) Induction of early mitotic events in a cell-free system. *Cell* 41:165–175.
- Nixon RA, Logvinenko KB (1986) Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed nonuniformly along axons of retinal ganglion cell neurons. *J Cell Biol* 102:647–659.
- Ottaviano Y, Gerace L (1985) Phosphorylation of the nuclear lamins during interphase and mitosis. *J Biol Chem* 260:624–632.
- Runge MS, El-Maghrabi MR, Claus TH, Pilgis SJ, Williams RC, Jr (1981) A MAP-2-stimulated protein kinase activity associated with neurofilaments. *Biochemistry* 20:175–180.
- Shaw G, Weber K (1983) The structure and development of the rat retina: an immunofluorescence microscopical study using antibodies specific for intermediate filament proteins. *Eur J Cell Biol* 30:219–232.
- Shecket G, Lasek RJ (1982) Neurofilament protein phosphorylation. *J Biol Chem* 257:4788–4795.
- Sihag RK, Nixon RA (1989) *In vivo* phosphorylation of distinct domains of the 70-kilodalton neurofilament subunit involves different protein kinases. *J Biol Chem* 264:457–464.
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Jujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76–85.
- Steinert PM, Roop DR (1988) Molecular and cellular biology of intermediate filaments. *Annu Rev Biochem* 57:593–625.
- Stewart JM, Young JD (1984) Solid phase peptide synthesis. Rockford, IL: Pierce Chemical.
- Tapscott SJ, Bennett S, Holtzer H (1981a) Neuronal precursor cells

- in the chick neural tube express neurofilament proteins. *Nature* 292: 836–838.
- Tapscott SJ, Bennett GS, Toyama Y, Klienbart F, Holtzer H (1981b) Intermediate filament proteins in the developing chick spinal cord. *Dev Biol* 86:40–54.
- Tora-Delbauffe D, Pierre M, Osty J, Chantoux F, Francon J (1986) Properties of neurofilament protein kinase. *Biochemistry* 23:283–289.
- Traub P, Vargias CE (1983) Involvement of the N-terminal polypeptide of vimentin in the formation of intermediate filaments. *J Cell Sci* 63:43–67.
- Vallano ML, Buckholz TM, DeLorenzo RJ (1985) Phosphorylation of neurofilament proteins by endogenous calcium/calmodulin-dependent protein kinase. *Biochem Biophys Res Commun* 130:957–963.
- Westwood JT, Church RB, Wagenaar EB (1985) Changes in protein phosphorylation during the cell cycle of Chinese hamster ovary cells. *J Biol Chem* 260:10308–10313.
- Wible BA, Smith KE, Angelides KJ (1989) Resolution and purification of a neurofilament-specific kinase. *Proc Natl Acad Sci USA* 86:720–724.
- Wong J, Hutchison SB, Liem RKH (1984) An isoelectric variant of the 150,000-dalton neurofilament polypeptide. *J Biol Chem* 259: 10867–10874.
- Yu, K-T, Czech MP (1984) Tyrosine phosphorylations of the insulin receptor β -subunit activates the receptor-associated tyrosine kinase activity. *J Biol Chem* 259:5277–5286.