Identification of Novel in Vitro PKA Phosphorylation Sites on the Low and Middle Molecular Mass Neurofilament Subunits by Mass Spectrometry†

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ABSTRACT: Phosphorylation of the head domains of intermediate filament proteins by second messenger-dependent kinases is important in regulating filament assembly. In the case of neurofilaments, head domain phosphorylation is known to be important in assembly, but few sites have been identified. Using matrix-assisted laser desorption−ionization (MALDI) and nano-electrospray mass spectrometry, we report the identification of several novel in vitro cAMP-dependent protein kinase (PKA) phosphorylation sites in the low (NF-L) and middle (NF-M) molecular mass neurofilament subunits. Neurofilament polypeptides were purified from adult rat brain, and fractions containing a mixture of NF-L and NF-M were nonradioisotopically phosphorylated with PKA prior to proteolytic digestion of the polypeptides in situ in polyacrylamide excised from SDS gels. Sites of phosphorylation were determined by mass spectrometric analysis of mixtures enriched in tryptic phosphopeptides. In NF-L, four novel sites were identified: serines 12, 41, and 49 in the head domain and serine 435 in the carboxyl-terminal tail domain, and data consistent with phosphorylation of serine 2 were obtained. Recombinant rat NF-L protein was also phosphorylated with PKA, and the same serines were identified as phosphorylation sites, with two additional sites, serine 43 and probable phosphorylation of serine 55. In NF-M, one novel site, serine 1 in the amino-terminal head domain, was found to be phosphorylated, and serine 46, also in the amino-terminal head domain, was confirmed as a PKA phosphorylation site.

Neurofilaments are class IV intermediate filament proteins associated with the neuronal cytoskeleton. In mature neurones, they consist of three subunits with apparent molecular masses, as determined by SDS−polyacrylamide gel electrophoresis (SDS−PAGE),[1] of 200 kDa (high-NF-H), 150 kDa (middle-NF-M), and 70 kDa (low-NF-L). The subunits are assembled into filaments with a diameter of approximately 10 nm, present in both axons and dendrites. Neurofilaments, together with other cytoskeletal elements, form a three-dimensional filamentous network that helps define neuronal shape and size, as well as participate in dynamic functions such as axoplasmic transport.

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[1] Abbreviations: PKA, cAMP-dependent protein kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactosidase; BSA, bovine serum albumin; nano-ES, nano-electrospray; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MALDI, matrix-assisted laser desorption−ionization; TOF, time-of-flight; CID, collision-induced dissociation; GSK-3α, glycogen synthase kinase 3α.

As with other classes of intermediate filament proteins, each subunit consists of three structural domains: an amino-terminal head domain, a highly conserved central α-helical rod domain, and a hyper-variable carboxyl-terminal tail domain. Neurofilaments are highly phosphorylated proteins, and experimental evidence indicates that there is continuous turnover of much of the phosphate (*1*−*3*). The posttranslational phosphorylation of neurofilament protein subunits appears to have roles in filament assembly, stability, plasticity, and transport in axons. In certain diseases, neurofilaments form abnormal accumulations which may be the result of defective assembly dynamics or transport of assembled neurofilaments, both of which are probably regulated by phosphorylation at specific sites on the proteins. Neurofilaments are the main component of neuronal inclusion bodies characteristic of some neurodegenerative conditions which include spheroids and Lewy-like bodies in amyotrophic lateral sclerosis (*4*−*6*), Lewy bodies in the substantia nigra in Parkinson's disease (*7*), and cortical Lewy bodies in dementia with Lewy bodies (*8*−*10*), and we have demonstrated that phosphorylation probably plays a crucial role in the organization of the network of assembled neurofilaments, the aberrant regulation of which may underlie inclusion body formation (*11*).

Phosphorylation of the N-terminal head domain of intermediate filament proteins has been shown to have a regulatory role in subunit assembly and disassembly. Phosphorylation of the head domain of vimentin, desmin, and glial fibrillary acidic protein by PKA or protein kinase C (PKC) in vitro prevents the assembly of subunits into filaments and

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causes assembled filaments to depolymerize (*12*−*14*). These effects can be reversed by dephosphorylation. Similarly, phosphorylation by PKA of the head domain of NF-L has been shown to block the assembly of isolated NF-L subunits into homopolymers and cause the disassembly of preexisting filaments in vitro (*15, 16*). Sihag and Nixon (*1*) identified serine 55 as a major PKA phosphorylation site in the head domain of NF-L; this site can incorporate phosphate in vivo and displays relatively rapid turnover of phosphate soon after NF-L synthesis. The cyclical phosphorylation and dephosphorylation of this site may regulate a step in neurofilament assembly and transport. Further in vivo evidence for the role of NF-L phosphorylation in regulating neurofilament assembly comes from the increase in neurofilament phosphorylation observed following treatment of dorsal root ganglion cultures with low nanomolar concentrations of the phosphatase inhibitor okadaic acid. Serine residues in the head domain of NF-L become phosphorylated, and rapid reversible disruption of the neurofilament network occurs under these conditions (*17, 18*). This implicates the involvement of the okadaic acid sensitive protein phosphatase-2A (PP-2A) in neurofilament dephosphorylation. Serine 2 on NF-L was identified as a phosphorylated site in these cultures and has been shown to be phosphorylated in vitro by PKA (*19*). It was suggested that this site is involved in the modulation of neurofilament dynamics through the antagonistic effects of PKA and PP-2A.

The N-terminal head domain of NF-M also contains sites that are targets for both PKA and PKC (*20*). Some of these sites exhibit early labeling and rapid turnover of phosphate similar to that observed for NF-L. Sihag and co-workers (*21*) have shown that serines 23, 28, 32, 41, and 46 are all PKA phosphorylation sites in vitro. The function of phosphorylation of the head domain of NF-M is not known but may also be important in regulating neurofilament assembly. There is evidence that the phosphorylation of either NF-L or NF-M by PKA is sufficient to inhibit their coassembly into filaments in vitro (*22*).

Phosphorylation at the carboxyl-terminal tail domains, particularly of NF-M and NF-H, has been implicated in the maintenance of axonal calibre (*23*) and in determining the rate of transport of neurofilaments in the axon (*24, 25*); neurofilaments in the cell soma and dendrites of neurones are much less phosphorylated in the tail domains of NF-H and NF-M (*2, 26*). The tail domains of these subunits contain multiple phosphorylation sites in repeated homologous sequence motifs (*27, 28*) which are phosphorylated by second messenger-independent protein kinases (*28*−*30*). However, there are putative phosphorylation sites in the tail domains that have not been shown to be phosphorylated by second messenger-independent kinases, one such site being serine 473 which has been identified as a phosphorylation site in the C-terminal tail of NF-L (*31*) both in vitro and in vivo, the kinase responsible remaining unidentified.

In view of the importance of phosphorylation in regulating various functions and properties of neurofilaments and the fact that the sites have not yet been completely identified, we have employed the sensitive technique of nano-electrospray mass spectrometry to identify sites in NF-L and NF-M that are phosphorylated in vitro by PKA, a physiologically important candidate neurofilament kinase. This technique also does not require prior ^{32}P-labeling of protein substrates. The in vivo sites of phosphorylation were previously identified in NF-L and NF-M purified from rat brain using mass spectrometry (*32*). Direct comparison of the mass spectrometric data from native rat brain polypeptides and the same material phosphorylated in vitro with PKA enabled us to identify additional PKA phosphorylation sites in NF-L and NF-M. Furthermore, we have phosphorylated full-length and truncated recombinant rat NF-L with PKA and shown that the same serine residues are phosphorylated as well as two additional sites.

It is important to identify sites that are phosphorylated in vitro because it is only then that the contribution of such sites to the physiological properties of neurofilaments can be established, for example, by assembly studies, both in vitro and in cells, of wild-type NF-L and NF-M and also NF-L and NF-M with the sites mutated so as to prevent phosphorylation. In this study, we have identified four novel sites in NF-L and one novel site in NF-M. We also confirm serine 2 of NF-L and serine 46 of NF-M as PKA phosphorylation sites. In recombinant NF-L, serines 43 and 55 were found to be phosphorylated by PKA in addition to the five sites in rat brain NF-L phosphorylated in vitro. Further studies will address the physiological function of these newly identified phosphorylation sites.

EXPERIMENTAL PROCEDURES

Materials. Except where otherwise noted, all chemicals were purchased from Sigma or Merck Ltd. and were of the highest quality available. Hydroxylapatite was from BioRad. Synthetic oligonucleotide primers were from Oswel DNA Service, University of Southampton, U.K. For mass spectrometric analysis, HPLC grade methanol and acetonitrile (Rathburn Chemicals, Scotland) were used.

Purification of Neurofilament Polypeptides. Intermediate filament proteins were isolated from adult rat brains as a Triton X-100-insoluble fraction. Approximately 15 g of tissue was homogenized in PBS (10 mM phosphate buffer, 140 mM NaCl, 2.7 mM KCl), pH 7.2, additionally containing 600 mM KCl, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 10 mM tetrasodium pyrophosphate, 20 mM sodium fluoride, 100 mM sodium orthovanadate, and 0.1% (v/v) Triton X-100. After centrifugation (Sorvall SS34 rotor, 21000g_{av}, 20 min, 4 °C), the insoluble pellet was washed once by rehomogenization in the same buffer but lacking Triton X-100 followed by recentrifugation and then dissolved in 8 M urea containing 10 mM sodium phosphate, pH 7.4, and 1 mM DTT. Batchwise hydroxylapatite chromatography (*33*) was used to separate neurofilament subunits from other intermediate filament proteins. Neurofilament-containing fractions were concentrated by dialyzing against 20 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 4 M urea and loading onto a Mono Q HR 5/5 column (Pharmacia Biotech); the column was eluted with a flow rate of 0.5 mL/min with a gradient of 0−1 M NaCl developed over 10 mL and monitored at 280 nm; 0.5 mL fractions were collected and analyzed by SDS−PAGE and Western blots.

Expression and Purification of Recombinant GST−*NF-L Fusion Proteins.* To facilitate the cloning of full-length and truncated rat NF-L cDNAs into the bacterial expression plasmid pGEX-3X (Pharmacia), a PCR strategy was employed whereby pairs of synthetic oligonucleotide primers

were used to introduce a *Bam*HI site at the translation start codon at the 5′ end and an *Eco*RI site at the 3′ end of each construct (Figure 1A). The parental plasmid pGEM-NFL cDNA (*34*) representing the full-length rat NF-L sequence was used to prime the synthesis of each of the PCR products encoding full-length NF-L (1629 bp), using primer 1 (5′CGTGGGATCCTCATGAGTTCGTTCAGCTAC3′) and primer 2 (5′CGATGAATTCCCTCAATCTTTCTTCTTAGC3′); head+rod domains of NF-L (1206 bp), using primer 1 and primer 3 (5′CGATGAATTCCCACTGAGCCTGGTTTCTTC3′); and only the head domain of NF-L (285 bp), using primer 1 and primer 4 (5′CGATGAATTCCCCTGCAGCTGTGCCTTCTC3′). The PCR products were subcloned into the pGEX-3X vector, and the DNA sequence of all three plasmid constructs was confirmed using the Cyclizt Exo$^-$ *Pfu* sequencing kit (Stratagene Ltd.). Electrocompetent *E. coli*, strain DH5α (Stratagene Ltd.), were transformed with the plasmid constructs or the pGEX-3X plasmid alone, and bacterial cultures were grown for 90 min at 30 °C, and then induced to express the fusion proteins with 0.1 mM IPTG for a further 4 h. The GST fusion proteins and GST alone were solubilized and purified according to the method of Frangioni and Neel (*35*). GST fusion proteins and GST alone were purified to homogeneity on glutathione−Sepharose 4B beads (*36*). The beads were washed 6−8 times in ice-cold PBS by repeated centrifugation and stored, with the protein remaining bound, at 4 °C.

Determination of Protein Concentration. The total protein concentration of mixed fractions of NF-L and NF-M was determined by the method of Bradford (*37*). The proportion of total protein in each subunit was determined by scanning a Coomassie-stained SDS gel using a Bio Image system and Whole Band Analyzer software. The protein concentration of the GST−NF-L fusion proteins was estimated by solubilizing the beads in SDS−PAGE sample buffer and comparing with BSA standards (0.25−10 mg/mL range) by dot-blot analysis.

In Vitro Phosphorylation. Fractions from the Mono Q HR 5/5 column containing a mixture of NF-L and NF-M (50 μg) were phosphorylated in vitro with the catalytic subunit of PKA (2 μg). Samples were first microdialyzed against 20 mM hepes, pH 7.5, containing 10 mM $MgCl_2$, 0.5 mM DTT. Phosphorylation was carried out in the same buffer with the addition of 10 μg/mL aprotonin, 5 μg/mL leupeptin, 7 μg/mL pepstatin, 0.1 μM sodium orthovanadate, 5 μM okadaic acid, 1 mM PMSF, and 3 mM ATP. The reaction was carried out overnight at 30 °C. GST−NF-L fusion proteins were phosphorylated while bound to the glutathione−Sepharose 4B beads; approximately 50 μg of full-length, head+rod, or head only fusion protein was phosphorylated in vitro with PKA (2 μg). Parallel incubations with [γ-^{32}P]ATP, containing the same concentrations of protein and kinase, were performed in order to detect phosphate incorporation by autoradiography, and in the case of the NF-L and NF-M purified from rat brain, to measure the stoichiometry of phosphate incorporation and for subsequent phosphoamino acid analysis. Radiolabeling experiments were carried out with the addition, to the above phosphorylation cocktail, of 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) (Dupont−New England Nuclear). Reactions were terminated by heating the samples to 100 °C for 5 min in SDS−PAGE sample buffer. ^{32}P incorporation into NF-L and NF-M was determined by Cerenkov counting of ^{32}P-labeled bands from SDS−PAGE.

SDS−PAGE. Samples from phosphorylation reactions were analyzed by 8% or 10% (w/v) acrylamide SDS−PAGE (*38*) and electrophoretically transferred to a poly(vinylidene difluoride) (PVDF) membrane using a Bio-Rad Transblot SD (*39*). Proteins were detected on the membrane by staining with 0.1% (w/v) amido black stain (*40*). Incorporation of ^{32}P was detected by autoradiography.

Phosphoamino Acid Analysis. Proteins separated by SDS−PAGE and transferred to PVDF membrane were excised after visualization with 0.1% (w/v) amido black stain and hydrolyzed in 5.7 M HCl at 110 °C for 1 h (*41*). The hydrolysate was lyophilized and resuspended in 2.2% (v/v) formic acid/7.8% (v/v) acetic acid, pH 1.9, containing 100 μg/mL of each phosphoamino acid standard: phosphoserine, phosphothreonine, and phosphotyrosine. Samples were spotted onto thin-layer cellulose plates (Merck Ltd.), phosphoamino acids were separated by electrophoresis in the first dimension at 1 kV for 30 min in pH 1.9 buffer and in the second dimension at 1 kV for 30 min in 10% (v/v) acetic acid, 1% (v/v) pyridine, pH 3.5. The phosphoamino acid standards were visualized with ninhydrin spray, and the position of the radiolabeled phosphoamino acids was determined by autoradiography.

Proteolysis of NF-L, NF-M, and GST−NF-L Fusion Proteins by in Situ Tryptic Digestion of SDS−PAGE Protein Bands. Following nonradioisotopic phosphorylation by PKA, approximately 50 μg of the mixture of NF-L and NF-M and 50 μg of each of the three NF-L fusion proteins were separated by one-dimensional SDS−PAGE (*38*) using precast 8% (w/v) acrylamide Novex (San Diego, CA) mini gels. Gels were stained with 0.2% (w/v) Coomassie Brilliant Blue R250 (CBB) in 50% (v/v) MeOH in water containing 2% (v/v) acetic acid and destained in 50% (v/v) MeOH, 2% (v/v) acetic acid. Protein bands were excised from the gel, reduced, carboxyamidated, and digested with trypsin (bovine sequencing grade: Boehringer Mannheim UK Ltd., East Sussex, U.K.) as described (*42*). Tryptic peptides were extracted by two changes of 100 mM NH_4HCO_3 and acetonitrile, followed by two changes of 5% (v/v) acetic acid and acetonitrile and concentrated to dryness by lyophilization in a Speed Vac (Savant).

Immobilized Metal Affinity Chromatography (IMAC). Miniature Protein Chemistry Systems (PCS) desalting columns (Hewlett-Packard, Cheshire, U.K.), supplied empty, were packed with Chelating Sepharose High Performance slurry [70 μL in 20% (v/v) ethanol; Pharmacia Biotech, Sweden] and washed with 2 mL of water, followed by 500 μL of 0.1 M acetic acid, pH 3.1 (solution A). Fifty microliters of 0.1 M $FeCl_3$ in solution A was applied, followed by washing with 500 μL of solution A to remove excess iron. The dried peptide mixture, dissolved in 50 μL of solution A, was loaded, and the column was then washed with 500 μL of solution A. The phosphopeptides were eluted with 300 μL of 0.1 M Tris-HCl, pH 8.5, and the eluate dried by lyophilization before being analyzed by mass spectrometry.

Matrix-Assisted Laser Desorption−Ionization (MALDI) Mass Spectrometry. MALDI mass spectrometry was performed on a VG TofSpec SE time-of-flight (TOF) mass

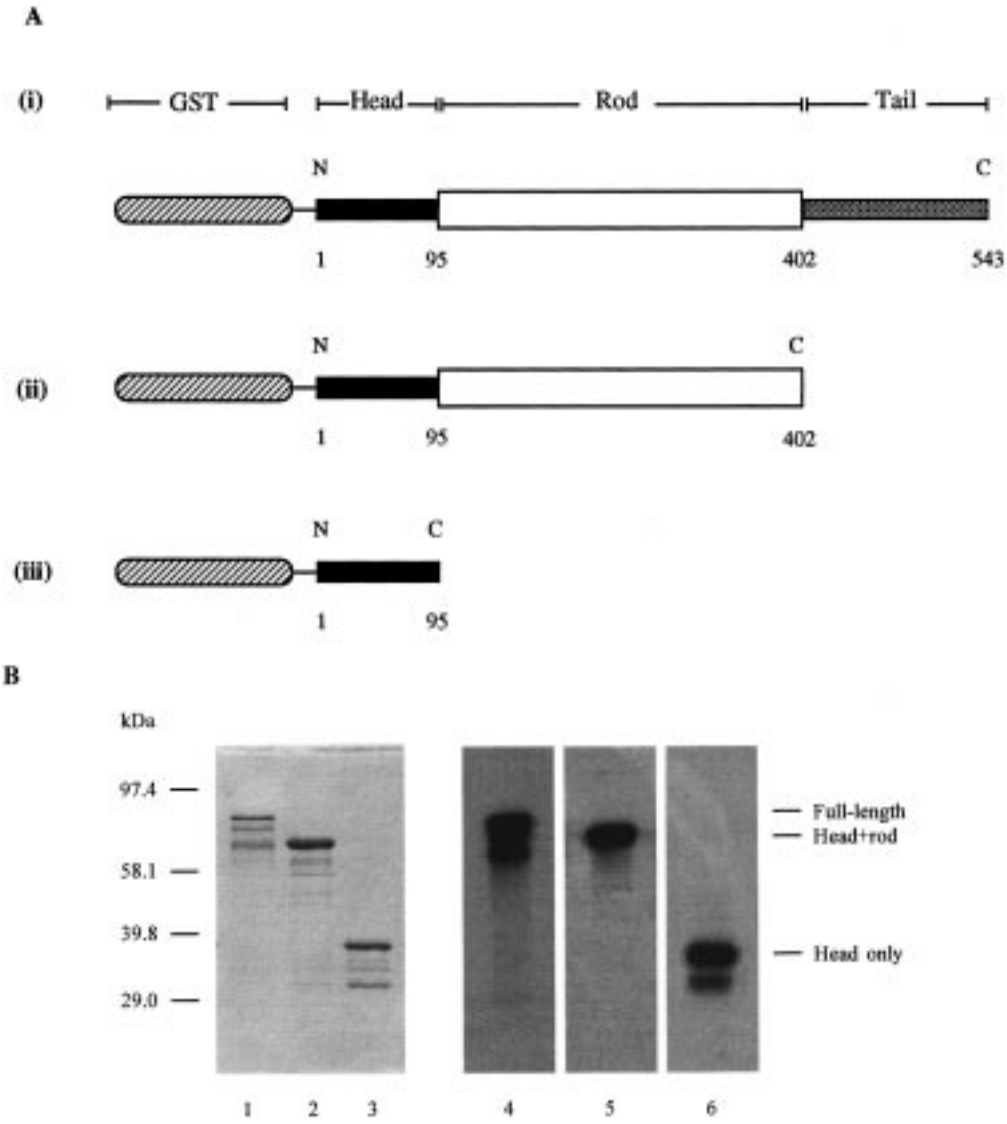

FIGURE 1: Expression and phosphorylation of recombinant GST−NF-L fusion proteins. (A) Schematic diagram illustrating the domains of NF-L represented in each of the fusion proteins: (i) full-length NF-L; (ii) NF-L head+rod; (iii) NF-L head only. NF-L amino acid residues are numbered. The short line between the GST and NF-L moieties in each fusion protein represents the linker peptide G-I-L-M. 20 μg of full-length NF-L, NF-L head+rod, or NF-L head only GST fusion proteins was phosphorylated, in the presence of [γ-^{32}P]ATP, with 1 μg of PKA in a total volume of 40 μL at 30 °C overnight. A 4 μL aliquot of each sample was subjected to 10% (w/v) acrylamide SDS−PAGE followed by autoradiography. (B) Lanes 1−3, Coomassie Blue staining of the gel; lane 1, full-length NF-L; lane 2, NF-L head+rod; lane 3, NF-L head only. Lanes 4−6, autoradiography of the same gel.

spectrometer equipped with a delayed extraction ion source and operating in the reflector mode (Micromass, Manchester, U.K.). A saturated solution of α-cyano-4-hydroxycinnamic acid in acetone was mixed in a 4:1 ratio (v/v) with a 10 g/L solution of nitrocellulose in acetone/2-propanol (1:1, v/v), and 0.6 μL aliquots of this solution were deposited on the stainless-steel target. Aliquots (0.5 μL) were taken from the tryptic digest mixtures of NF-L, NF-M, and the NF-L fusion proteins and loaded onto the target as described (*43*). Samples were air-dried and washed with 10 μL of 5% (v/v) formic acid, followed by 10 μL of water, prior to insertion into the instrument. Spectra were externally calibrated against a standard peptide mix (Substance P, 1347.74; Neurotensin, 1672.92; ACTH fragment 2465.20) and monoisotopic masses assigned. Instrument parameters were set as follows: frequency, 500 mHz; sensitivity, 50 mV; source voltage, 20 000 V; extraction voltage, 19 874 V; focus voltage, 16 448 V; reflectron voltage, 25 000 V; detector, 1600 V. A resolution of 4000 was typically gained on peaks at *m*/*z* 2000.

Nano-electrospray (Nano-ES) Mass Spectrometry (MS). Needles for nano-electrospray mass spectrometry were made with a micropipet puller (Sutter Instrument Co., Novato, CA) from borosilicate glass capillaries (Clark Electromedical Instruments, Pangbourne, Reading, U.K.) as described by Wilm and Mann (*44*). They were gold-coated in a vapor desorption instrument. Dried protein digests were dissolved in 0.5% (v/v) formic acid and desalted on a PCS column self-packed with ~10 μL of POROS sorbent (PerSeptive Biosystems, Cambridge, MA) by washing with 200 μL of 0.5% (v/v) formic acid and eluting with 100 μL of 50% (v/v) MeOH, 5% (v/v) formic acid. The desalted sample was again dried by lyophilization and taken up in 5 μL of spraying solution [50% (v/v) MeOH, 1% (v/v) formic acid in water for positive ion or 50% (v/v) MeOH, 5% (v/v) ammonia in water for negative ion], and 1 μL was inserted

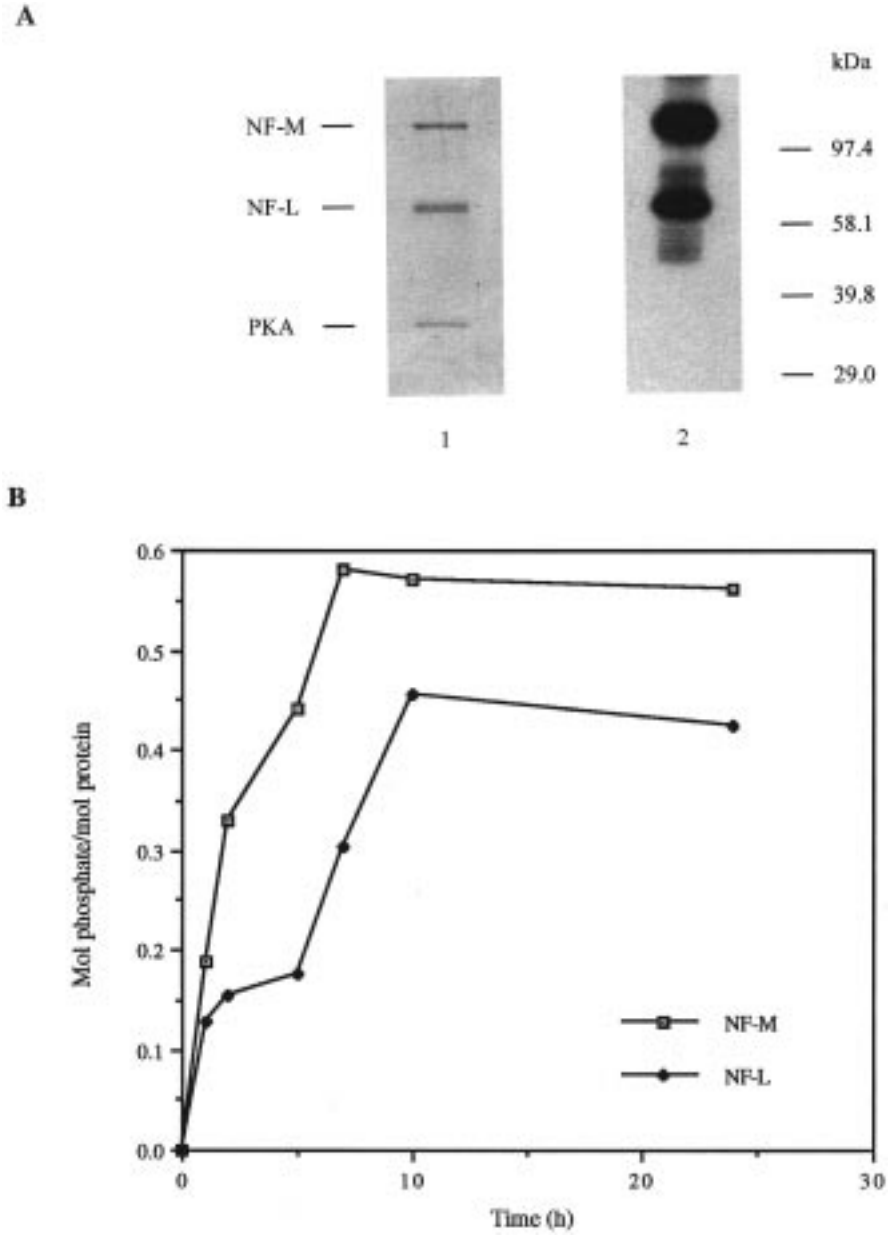

FIGURE 2: Stoichiometry of phosphorylation of NF-L and NF-M. A mixture of NF-L (10 μg) and NF-M (10 μg) isolated from rat brain was phosphorylated, in the presence of [γ-^{32}P]ATP, with 1 μg of PKA in a total volume of 40 μL at 30 °C, and aliquots were removed at the various times indicated. A 4 μL aliquot of the labeled sample was taken after 24 h and subjected to 8% (w/v) acrylamide SDS−PAGE followed by autoradiography (A). Lane 1, Coomassie Blue staining of the gel; lane 2, autoradiography of the same gel. Incorporation of ^{32}P at each time was determined as described under Experimental Procedures and plotted as a graph (B).

into the spraying needle. Electrospray mass spectra were acquired on an API III triple quadrupole instrument (PE-Sciex, Ontario, Canada) equipped with a nano-ES ion source developed by Wilm and Mann (*44*, *45*). Q_1 scans were performed with 0.1 Da mass steps. For operation in the MS/MS mode, Q_1 was set to transmit a mass window of 2 Da for both parent and product ion scans, and spectra were accumulated with 0.2 Da mass steps. The dwell time was 1 ms for all scans except for parent ion scans, where it was 3 ms. Resolution was set to a value of 121 for Q_1 scans, 116 for parent ion scans, and 113 for product ion scans so that masses could be assigned to better than 1 Da. Collision energy was tuned individually for each peptide for optimum MS/MS spectra. A new needle was used for each experiment. Spectra interpretation was performed using BioMultiView (Sciex) software.

RESULTS

Expression, Purification, and Phosphorylation of Recombinant GST−NF-L Fusion Proteins. Recombinant full-length and truncated NF-L were expressed as GST fusion proteins in *E. coli* and purified by affinity chromatography on glutathione−Sepharose 4B beads; the yield of each protein was 1−2 mg/L of bacterial culture. The mass of the full-length NF-L fusion protein was 84 kDa (Figure 1B, lane 1), NF-L head+rod fusion protein, 69 kDa (Figure 1B, lane 2), and NF-L head only fusion protein, 36 kDa (Figure 1B, lane 3); the GST moiety represents 26 kDa of these molecular masses. A short linker peptide, glycine-isoleucine-leucine-methionine, encoded by the vector, separates the GST and NF-L moieties in each fusion protein. Control phosphorylation experiments with PKA and [γ-^{32}P]ATP were con-

FIGURE 3: Sequence coverage obtained and phosphorylation sites identified by MALDI and nano-ES mass spectrometry of PKA-phosphorylated rat brain NF-L, recombinant NF-L fusion proteins, and rat brain NF-M. (A) Amino acid sequence of rat brain NF-L showing 64% sequence coverage. (B) Amino acid sequence of the full-length recombinant NF-L showing 81% sequence coverage. (C) Amino acid sequence of rat brain NF-M showing 51% sequence coverage. Peptides identified by either MALDI or nano-ES are underlined; phosphorylation sites identified by nano-ES sequencing are in boldface type. Ac, acetyl group.

ducted with GST alone; no incorporation of ^{32}P was detected (not shown). Incubation of the recombinant fusion proteins with PKA in the presence of [γ-^{32}P]ATP, as described under Experimental Procedures, resulted in incorporation of phosphate, which was detected by autoradiography (Figure 1B, lanes 4−6).

PKA Phosphorylation of Rat Brain NF-L and NF-M. Incubation of a mixture of NF-L and NF-M purified from rat brain (Figure 2A) with catalytic amounts of PKA, in the presence of [γ-^{32}P]ATP, as described under Experimental Procedures, resulted in a time-dependent incorporation of approximately 0.5 mol of phosphate/mol of NF-L and 0.5 mol of phosphate/mol of NF-M (Figure 2B). Phosphoamino acid analysis of ^{32}P-labeled NF-L and NF-M phosphorylated by PKA revealed that the kinase phosphorylates both neurofilament subunits predominantly on serine residues (data not shown).

Identification of Phosphopeptides. A mixture of NF-L and NF-M from rat brain and individual GST−NF-L fusion proteins nonradioisotopically phosphorylated by PKA were separated on SDS−PAGE; the bands were excised and the proteins digested in situ with trypsin. Analysis of an aliquot of each digest by MALDI-MS gave a peptide mass map of each protein covering 23% of the sequence of NF-L and 30% of the sequence of NF-M from the rat brain preparation. MALDI analysis of the NF-L fusion proteins resulted in 89%

Table 1: Phosphopeptides Identified in the Tryptic Digest of PKA-Phosphorylated Rat Brain NF-L[b]

Peptide	Residues	Sequence	Average Mass (Da)	Site(s) Identified
L1	422-437	SAYSGLQSSSYLMS$_p$AR	1787.9	Serine **435**
L2	1-14	AcSS$_p$FSYEPYFSTSYK[a]	1814.8	Serine **2**
L3	37-53	SAYSS$_p$YSAPVSSSLSVR	1827.9	Serine **41**
L4	37-53	SAYSS$_p$YSAPVSSS$_p$LSVR	1907.8	Serine **41, 49**
L5	1-15	AcSS$_p$FSYEPYFSTSYKR[a]	1971.0	Serine **2**
L6	37-54	SAYSS$_p$YSAPVSSSLSVRR	1984.0	Serine **41**
L7	1-15	AcSS$_p$FSYEPYFSTS$_p$YKR[a]	2051.0	Serine **2, 12**
L8	37-54	SAYSS$_p$YSAPVSSS$_p$LSVRR	2064.0	Serine **41, 49**

[a] Phosphorylation site assignment based on findings of Giasson et al. (*19*) and site identified here on recombinant NF-L. [b] Phosphopeptides identified within the PKA-phosphorylated NF-L digest are shown. Fragmented peptides have residues found as a continuous Y″ or b ion series underlined. The phosphorylated residues are shown in boldface. Ac, acetyl group.

sequence coverage for the head domain, 59% for the head plus rod domains, and 81% for the full-length recombinant NF-L. Half of the digest mixtures of NF-M and NF-L isolated from rat brain were desalted on POROS and analyzed directly by nano-ES tandem mass spectrometry (MS/MS). Comparison of spectra from the PKA-phosphorylated material with those from protein which had not been phosphorylated in vitro (*32*) revealed many common ions. These, in combination with the MALDI data, gave sequence coverage of 64% for NF-L and 51% for NF-M (Figure 3).

Phosphopeptides were isolated from the remainder of the digest mixtures of PKA-phosphorylated rat brain NF-L and NF-M and from the PKA-phosphorylated recombinant NF-L fusion protein digests using a small-scale IMAC technique, which takes advantage of the affinity of phosphopeptides for immobilized Fe^{3+} ions (*46*). Following small-scale desalting, the samples were analyzed by nano-ES MS/MS, and phosphopeptides were identified using scans for the parents of *m*/*z* 79 in the negative ion mode (*47*, *48*) and for neutral loss of *m*/*z* 49, $[M-H_3PO_4+2H]^{2+}$, in the positive ion mode (*49*). The parent ion scan identifies ions which fragment to produce an ion at *m*/*z* 79 (the phospho group, PO_3^-). The neutral loss scan shows the masses of precursor ions which lose the phosphate group as a neutral fragment.

These ions were then fragmented in the positive ion mode in order to characterize the peptide and site of phosphorylation. Phosphopeptides were more easily identified and sequenced in the IMAC-purified samples. This is because the use of the IMAC column reduces the problem of electrospray ion suppression by reduction of the complexity of the peptide mixture (*32*, *47*, *50*). This reduction increases the phosphorylated peptide ion signal strength and thus improves the quality of the product ion spectra.

PKA Sites Identified on Rat Brain NF-L. Eight phosphopeptides corresponding to five discrete phosphorylation sites on NF-L (serines 2, 12, 41, 49, 435), phosphorylated in the presence of NF-M, were detected in the IMAC-separated sample (Table 1). None of these sites were previously identified in the native NF-L sample isolated from rat brain (*32*) and therefore must have been phosphorylated by PKA in vitro. Four of these sites (serines 2, 12, 41, 49) are situated within the N-terminal head region of NF-L where PKA has previously been shown to phosphorylate neurofilaments. The fifth site (serine 435) is located in the C-terminal tail domain where no previous instance of phosphorylation by PKA has been reported. The unphosphorylated species of all these peptides were also seen within the digest mixture indicating incomplete incorporation of phosphate by PKA.

Figure 4 shows the identification of phosphopeptides by the use of neutral loss scans in the positive ion mode (Figure 4A) and parents of *m*/*z* 79 scans in the negative ion mode (Figure 4B). The peptides and sites of phosphorylation were then characterized by collision-induced dissociation (CID) tandem mass spectrometry in the positive ion mode, resulting in product ion spectra. Sequence tags were constructed from the resulting fragment ion masses and hence peptides identified from the published sequence (*34*, *51*).

Fragmentation of the 2+ ion of L1 gave Y″ ions corresponding to the residues ..SGLQSS.. (Figure 5A), identifying the peptide as residues 422−437 with one phosphate added (SAYSGLQSSSYLMSAR, M_r 1787.9) and indicating that the phosphate is present on either serine 431 or serine 435. A series of b ions relating to the sequence tag ..LM.. indicate that the phosphate is in fact on serine 435 since the masses of the b ions equate to residues 422−432 with no phosphate added.

MS/MS of the 2+ ion of L2 resulted in a series of Y″ ions giving the partial sequence ..SYE.. (data not shown) and confirmed the phosphopeptide as AcSSFSYEPYFSTSYK, residues 1−14 (M_r 1814.8), with a phosphate on either serine 1 or serine 2. L5, corresponding to the missed cleavage product (residues 1−15, M_r 1971.0), fragmented to give the same sequence tag. From the data obtained, it cannot be unequivocally stated which serine carries the phosphate. However, since serine 2 on NF-L in okadaic acid-treated rat dorsal root ganglia cultures has been reported to be phosphorylated by PKA (*19*), and we have directly identified serine 2 as a PKA phosphorylation site on recombinant NF-L studied here (Table 2), it is perhaps more likely that this is the phosphorylated residue.

Fragmentation of L7, corresponding to residues 1−15 with two serines phosphorylated (M_r 2051.0), resulted in ions indicating that serine 12 was phosphorylated, these being Y_4'', representing the fragment ion $[^{12}S_pYKR]^+$, and Y_7'',

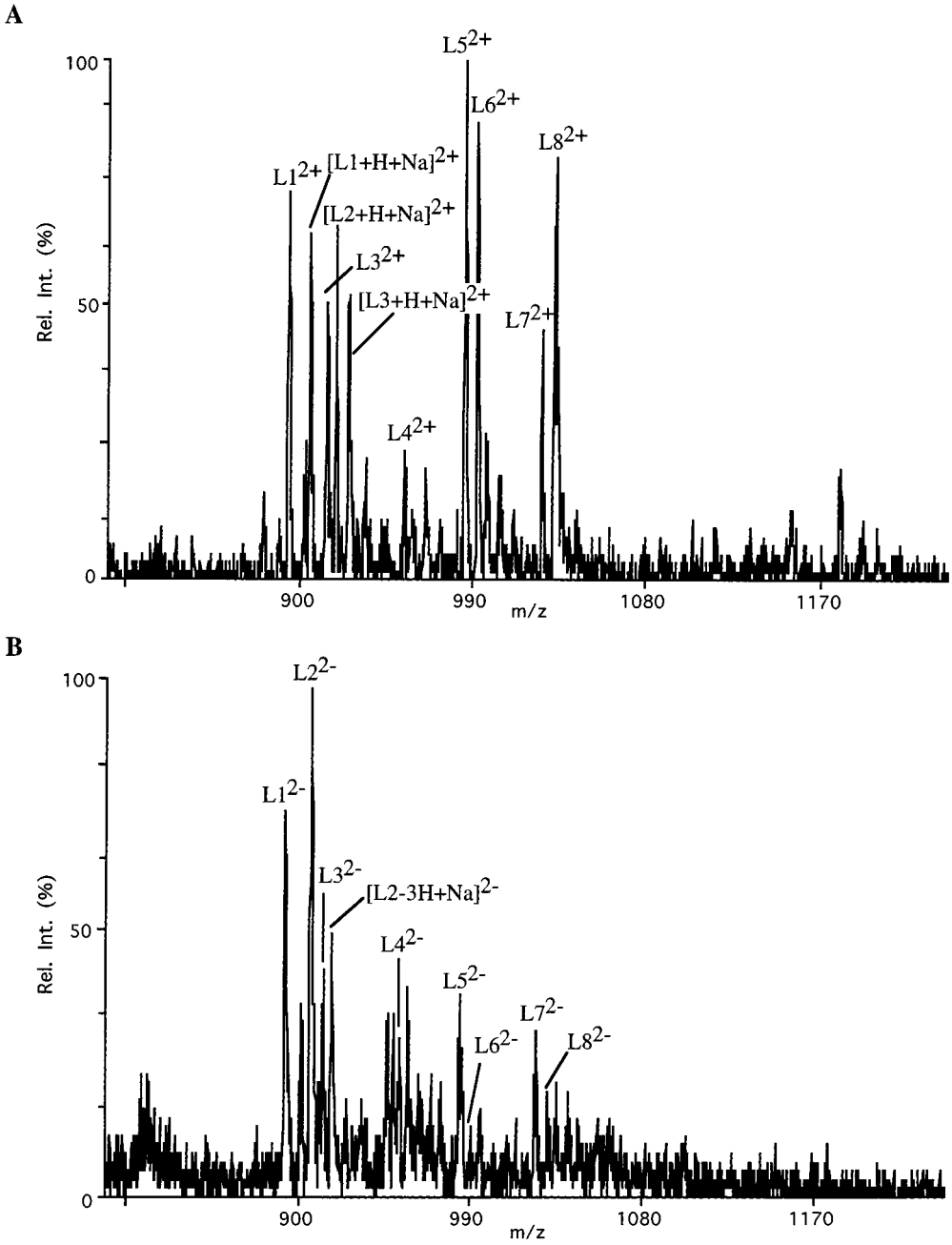

FIGURE 4: Identification of phosphopeptides in PKA-phosphorylated rat brain NF-L tryptic digest. (A) Scan for neutral loss of the phosphate group in the positive ion mode for PKA-phosphorylated NF-L. This shows the masses of precursor ions which lose the phosphate group as a neutral fragment, $[M-H_3PO_4+2H]^{2+}$. (B) Parent ion scan for the phospho group in the negative ion mode of PKA-phosphorylated NF-L. This shows the masses of ions which fragment to produce an ion at m/z 79 (PO_3^-).

representing the fragment ion $[FST^{12}S_pYKR]^+$. Fragment ions corresponding to the loss of proline 7 (Y_8'' and Y_9'') were also present. As in the case of the singly phosphorylated N-terminal peptide, it is impossible to tell from the data whether serine 1, 2, or 4 carries the other phosphate, but again since serine 2 has previously been identified as a phosphorylation site and from the results of our studies of PKA-phosphorylated recombinant NF-L (Table 2), we propose that it is also serine 2 that represents the second site in peptide L7.

The product ion spectrum of the 2+ ion of L3 displayed a series of Y″ ions relating to the sequence ..$S^{41}S_pYSA$.., thereby identifying the peptide as residues 37−53 and demonstrating that serine 41 is phosphorylated (SAY$S^{41}S_p$-YSAPVSSSLSVR, M_r 1827.9).

The same peptide with two sites phosphorylated is represented by L4 (M_r 1907.8). Fragmentation resulted in a continuous series of Y″ ions covering the sequence ..SAPVS$S^{49}S_p$LSVR (Figure 5B) and indicating that serine 49 is phosphorylated and the remaining phosphoserine lies within residues 37−42. b ions corresponding to residues 39 and 40 (..YS..) are also present and hence indicate that it is serine 41 which carries the phosphate, in accordance with the singly phosphorylated peptide. The missed cleavage products (residues 37−54) of both the singly and doubly phosphorylated peptides were also seen in the neutral loss and parents of *m/z* 79 scans (Figure 4, L5, M_r 1984.0; and L8, M_r 2064.0). MS/MS of the 2+ ion of L5 resulted in fragments confirming serine 41 as the site of phosphorylation on the singly phosphorylated peptide. The signal-to-noise

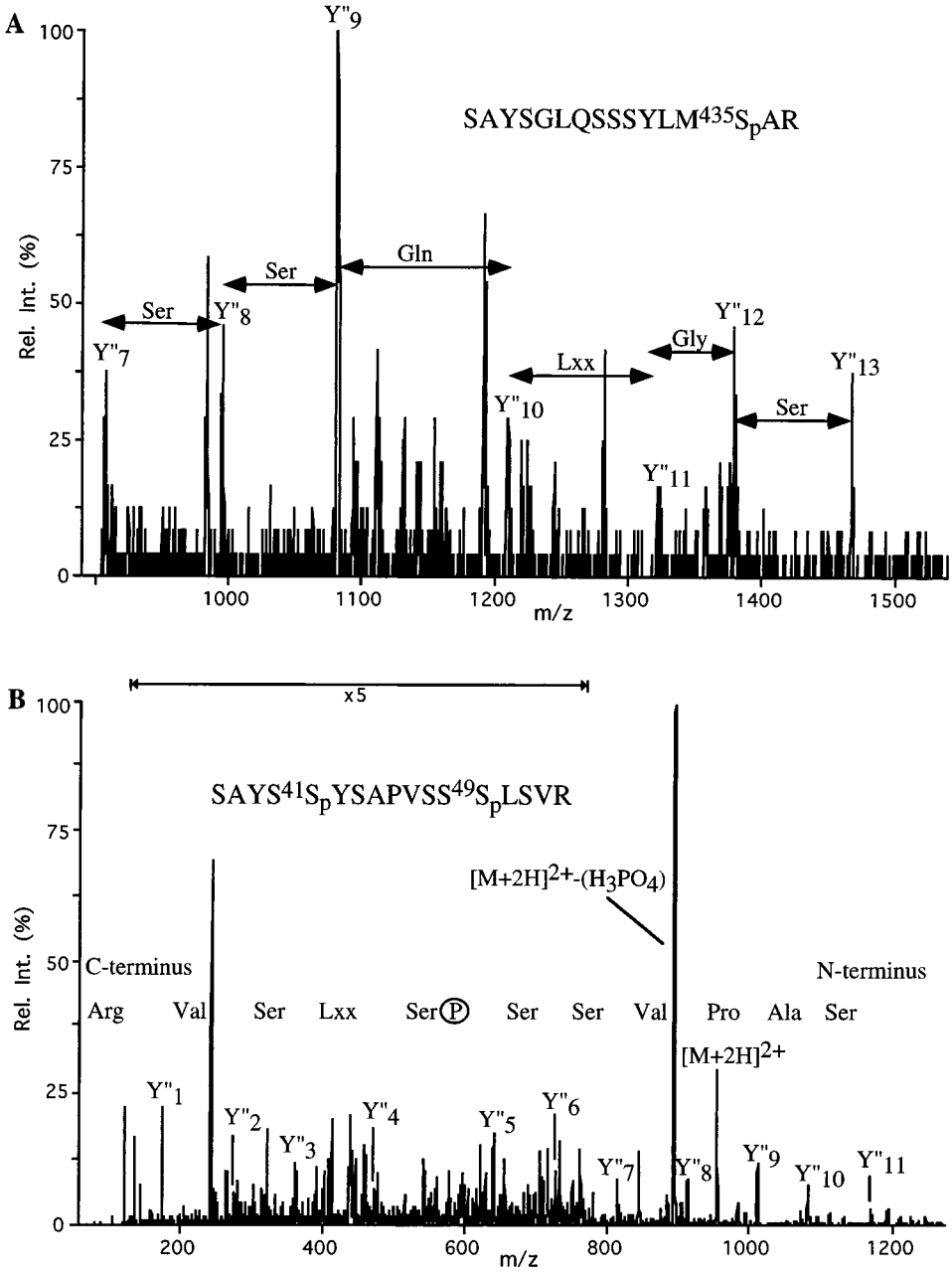

FIGURE 5: Tandem mass spectrometry of rat brain NF-L PKA-phosphorylated peptides. (A) Part of the positive ion tandem mass spectrum of L1 (SAYSGLQSSSYLMS$_p$AR) showing the Y″ ion series. (B) Positive ion tandem mass spectrum of L4 showing the Y″ ion series identifying the phosphopeptide as SAYSS$_p$YSAPVSSS$_p$LSVR.

ratio of the CID spectrum of L8 was not sufficient to produce any useful sequence information.

PKA Sites Identified on GST−NF-L Fusion Proteins. Nano-electrospray analysis of the three NF-L fusion proteins resulted in confirmation of the sites found to be phosphorylated by PKA on NF-L isolated from rat brain; serines 12, 41, and 49 [numbering as for native rat NF-L, (*34*)] on all three fusion proteins and, additionally, serine 435 on the full-length recombinant NF-L (Table 2).

In addition, serine 2 was directly confirmed as a site of phosphorylation by tandem mass spectrometry of the N-terminal peptide (*m*/*z* 1094.4). This resulted in a series of Y″ ions corresponding to the partial sequence S^{2}S$_p$-FSYEPYFSTSYK and identifying the peptide as GILMS^{2}S$_p$-FSYEPYFSTSYK (M_r 2187.3) with serine 2 phosphorylated.

A singly phosphorylated peptide corresponding to residues 55−83 (SYSSSSGSLMPSLENLDLSQVAAISNDLK, M_r 3094.3) was present in all three fusion protein samples but not in NF-L phosphorylated with PKA in the presence of NF-M. Fragmentation of this peptide (*m*/*z* 1548.0) resulted in a series of Y″ ions corresponding to the partial sequence PSLENLDLSQVAAIS (Figure 6). The data obtained were insufficient to locate the exact site of phosphorylation, although from the fragment masses one of the serines within the first eight residues must carry the phosphate. Since serine 55 is the only one of these residues to have been previously identified as a PKA phosphorylation site on NF-L (*1*), it is most likely that this is the phosphorylated residue.

Fragmentation of the peptide corresponding to residues 37−53 with one phosphate (M_r 1827.8) revealed two Y″ ion series corresponding to two differentially phosphorylated species in each of the fusion protein samples. These corresponded to phosphorylation at either serine 41 or serine 43.

Table 2: Phosphopeptides Identified in the Tryptic Digest of PKA-Phosphorylated Recombinant NF-L[b]

Peptide	Residues	Sequence	Average Mass (Da)	Site(s) Identified
R1	422-437	SAYSGLQSSSYLMS$_p$AR	1787.9	Serine **435**
R2	1-14	GILMSS$_p$FSYEPYFSTSYK	2187.3	Serine **2**
R3	37-53	SAYSS$_p$YSAPVSSSLSVR	1827.9	Serine **41**
R3	37-53	SAYSSYS$_p$APVSSSLSVR	1827.9	Serine **43**
R5	1-15	GILMSS$_p$FSYEPYFSTSYKR	2343.5	Serine **2**
R6	37-54	SAYSSYSAPVSSS$_p$LSVRR	1984..0	Serine **49**
R7	1-15	GILMSS$_p$FSYEPYFSTS$_p$YKR	2423.5	Serine **2, 12**
R9	55-83	S$_p$YSSSSGSLMPSLENLDLSQVAAISNDLK[a]	3094.3	Serine **55**

[a] Phosphorylation site assignment based on findings of Sihag and Nixon (*1*). Peptide numbers correspond to those of peptides identified in rat brain NF-L. [b] Phosphopeptides identified within the PKA-phosphorylated NF-L fusion protein digests are shown. Fragmented peptides have residues found as a continuous Y″ or b ion series underlined. The phosphorylated residues are boldface.

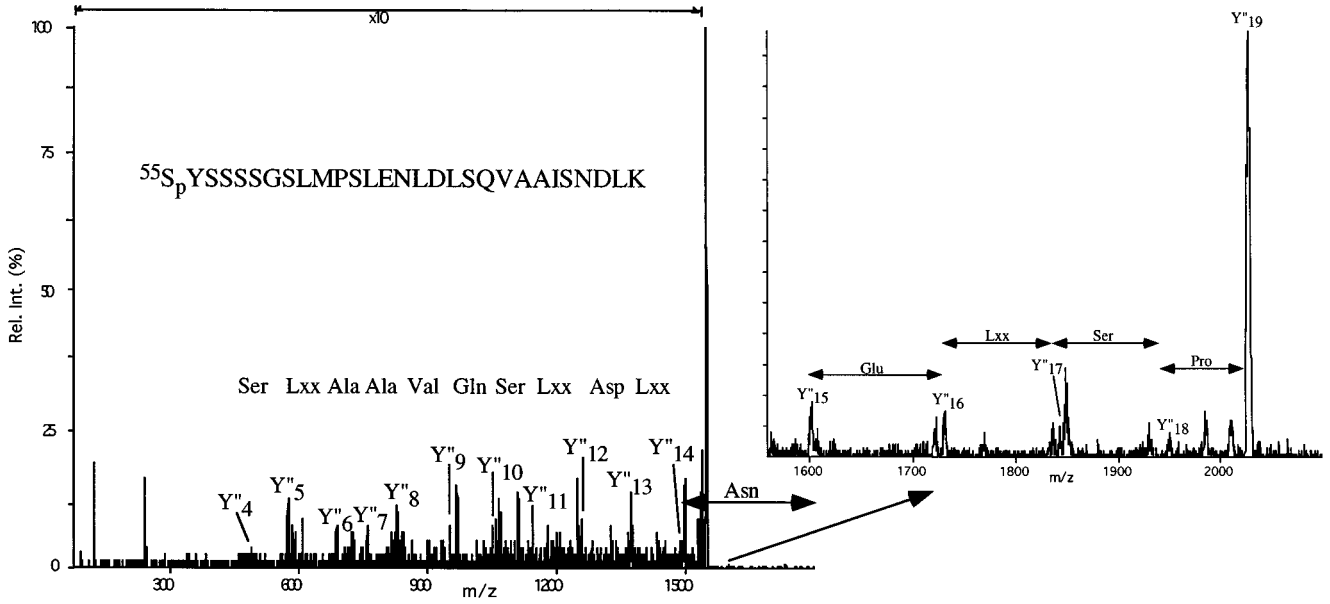

FIGURE 6: Tandem mass spectrometry of PKA-phosphorylated NF-L fusion protein peptides. Part of the positive ion tandem mass spectrum of peptide R9 (S$_p$YSSSSGSLMPSLENLDLSQVAAISNDLK) showing the Y″ ion series.

PKA Sites Identified on Rat Brain NF-M. Parents of *m*/*z* 79 and neutral loss of 49 scans revealed five phosphopeptides corresponding to phosphorylation at serines 603, 608, 666, and 766, already characterized as endogenous sites within the NF-M molecule (Table 3, *28*, *32*). Two other phosphopeptides, corresponding to two N-terminal phosphorylation sites (serines 1 and 46), not found in the protein analyzed without in vitro phosphorylation (*32*) were identified (Table 3) and must therefore have been phosphorylated by PKA *in vitro*. In vitro phosphorylation of serine 46 by PKA in mouse NF-M has been previously reported by Sihag and coworkers (*21*). They also identified four additional PKA sites in the N-terminal head domain; however, serine 1, identified here as being phosphorylated, was not reported. As in the case of NF-L, both peptides identified were also seen in their unphosphorylated forms, again indicating incomplete incorporation of phosphate by PKA.

Neutral loss (Figure 7A) and parent ion scans (not shown) were used to identify peptides phosphorylated by PKA in NF-M. These were then fragmented by CID tandem mass spectrometry, enabling the peptide and site of phosphorylation to be determined from the published sequence (*52*). The 2+ ion of M2 was fragmented to give a series of Y″ ions (Figure 7B) corresponding to the residues ..^{46}S$_p$TVSSSYKR and characterizing the peptide as GSP^{46}S$_p$TVSSSYKR, residues 43−54 with serine 46 phosphorylated (M_r 1335.3). Fragmentation of M4 gave Y″ ions relating to the sequence ..LDSL... A strong Y″$_5$ ion (data not shown) corresponding to the fragment [PSAYR]$^+$ was also seen in the spectrum. This ion is expected to be strong since cleavage at the N-terminal amide bond of proline is highly favored. The masses of these fragments characterize the peptide as Ac^{1}S$_p$-YTLDSLGNPSAYR, residues 1−14, M_r 1665.7, and indicate that serine 1 carries the phosphate. The presence of b$_{10}$, corresponding to the fragment [AcS$_p$YTLDSLGNP]$^+$, confirms that serine 1 is phosphorylated.

DISCUSSION

Conventional approaches used to study the phosphorylation state of neurofilament proteins have, until recently, required

Table 3: Phosphopeptides Identified in the Tryptic Digest of PKA-Phosphorylated Rat Brain NF-M[a]

Peptide	Residues	Sequence	Average Mass (Da)	Site(s) Identified
M1	663-671	KAES$_p$PVKEK	1095.1	Serine **666**
M2	43-54	GSPS$_p$TVSSSYKR	1335.3	Serine **46**
M3	757-771	GVVTNGLDVS$_p$PAEEK	1594.6	Serine **766**
M4	1-14	AcS$_p$YTLDSLGNPSAYR	1665.7	Serine **1**
M5	603-620	SPVPKS$_p$PVEEVKPKPEAK	2026.2	Serine **608**
M6	601-620	AKSPVPKS$_p$PVEEVKPKPEAK	2225.4	Serine **608**
M7	601-620	AKS$_p$PVPKS$_p$PVEEVKPKPEAK	2305.3	Serine **603, 608**

[a] Phosphopeptides identified within the PKA-phosphorylated NF-M digest are shown. Fragmented peptides have residues found as a continuous Y″ or b ion series underlined. The phosphorylated residues are shown in boldface. Ac, acetyl group.

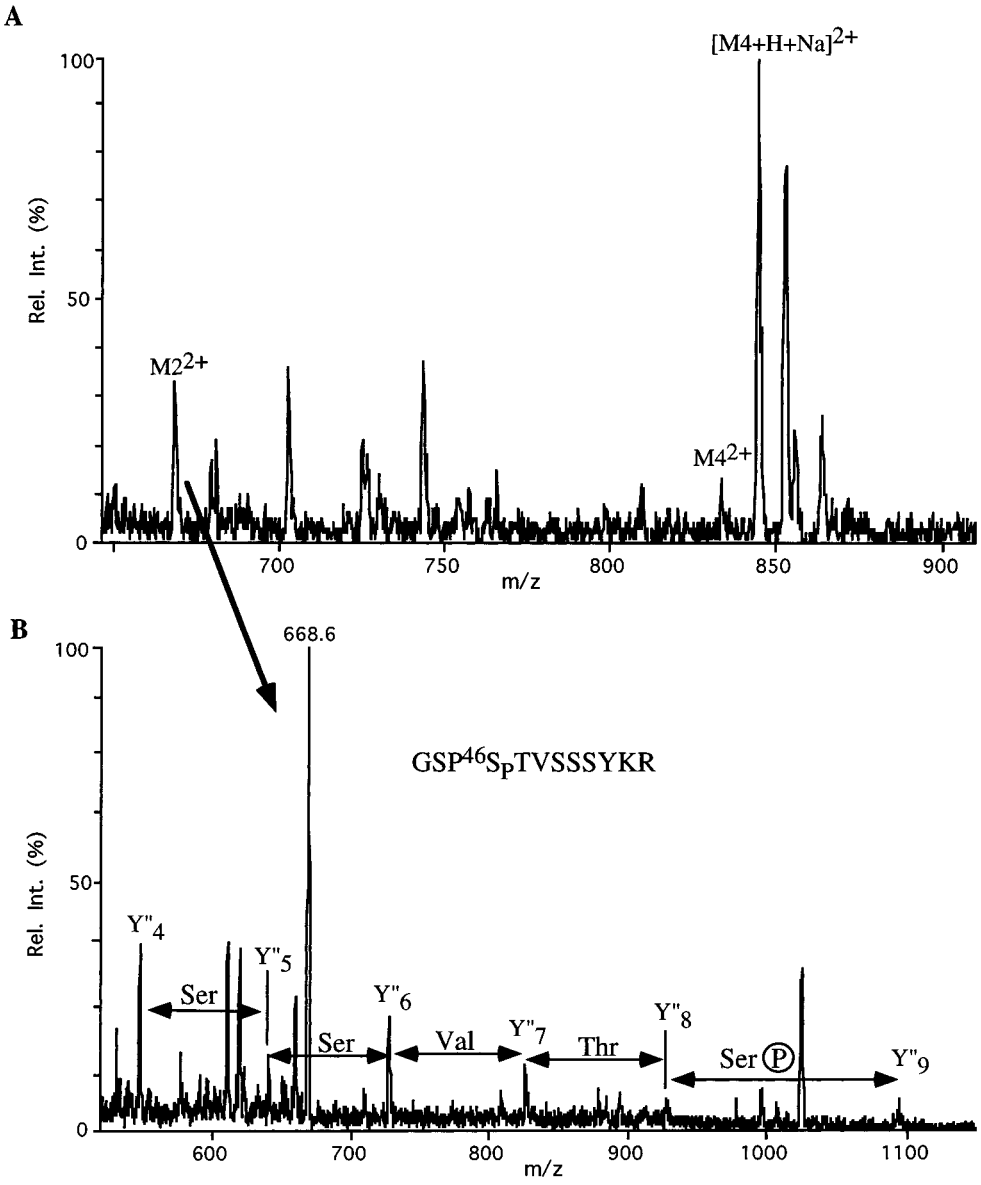

FIGURE 7: Identification and characterization of phosphopeptides in PKA-phosphorylated rat brain NF-M tryptic digest. (A) Scan for neutral loss of the phosphate group positive ion mode for PKA-phosphorylated NF-M. This shows the masses of precursor ions which lose the phosphate group as a neutral fragment, $[M-H_3PO_4+2H]^{2+}$. (B) Part of the positive ion tandem mass spectrum of M2 ($GSPS_pTVSSSYKR$) showing the sequence tag and phosphopeptide identified.

the purification of the neurofilament subunits to homogeneity and chromatographic separation of enzymatic digest mixtures and ultimately have been limited by the sensitivity of Edman degradation sequencing. In this report, we identify specific

sites of PKA phosphorylation by nano-electrospray mass spectrometric sequencing of proteins direct from polyacrylamide gels. This technique eliminates the need for subunit separation and enables sequencing of peptides from a total digest mixture at higher sensitivity levels. The nature of the fragments generated by tandem MS of peptides allows unique characterization of the peptide from the known protein sequence. A further advantage of the technique is that a radioisotopic label is not required; this is the first example of the identification of phosphorylation sites of proteins phosphorylated in vitro in the absence of a radioisotopic phosphate label.

We compared our previously published mass spectrometric data from isolated native rat brain NF-L and NF-M in which we identified the in vivo phosphorylation sites (*32*), with data obtained here from the same material phosphorylated in vitro with PKA. Hence, we were able to identify those additional sites that were targeted by PKA. In addition, we phosphorylated recombinant NF-L proteins with PKA, and since bacterially expressed proteins are not posttranslationally modified and therefore do not contain endogenous phosphate, we were able to compare the mass spectrometric data from three sources of NF-L and two sources of NF-M to aid in the interpretation of our results.

Using this approach, we have characterized five PKA phosphorylation sites in NF-L (Table 1). One of these sites, serine 2, has been reported previously (*19*), but we were able to identify three novel sites (serines 12, 41, 49) in the amino-terminal head domain of NF-L. In addition, we have shown that PKA can also phosphorylate the carboxyl-terminal tail domain at serine 435; this is the first report of the phosphorylation of a specific site in the tail domain of a neurofilament subunit by a second messenger-dependent protein kinase.

We used as substrate a mixture of NF-L and NF-M that had been renatured by dialysis to remove urea and hence had most likely assembled to form oligomers since the conditions were similar to those reported to promote filament assembly from NF-L plus NF-M (*22, 53*). Under these conditions, we found the stoichiometry of phosphate incorporation by PKA to be approximately 0.5 mol of phosphate/mol of NF-L (Figure 2B). Previous assays of the stoichiometric incorporation of phosphate into NF-L by PKA have given figures of up to 4 mol of phosphate/mol of NF-L for various sources of NF-L (*22, 53*). Since we used a mixture of NF-L and NF-M, sterically hindered access of PKA to certain sites may have resulted in a reduced stoichiometry. This seems to be a likely explanation since serines 43 and 55 were not found as phosphorylated sites in NF-L isolated from rat brain (*32*), nor were they phosphorylated in the renatured NF-L plus NF-M mixture, but were phosphorylated in the recombinant NF-L fusion proteins. Serine 55 has been shown to be phosphorylated transiently in vivo with dephosphorylation being associated with incorporation of newly synthesized NF-L into assembled neurofilaments (*1*); phosphorylation of serine 43 may therefore be similarly regulated.

An alternative explanation for the difference in stoichiometry between our data and those of others may be because others used phosphatase-treated NF-L (*53*), hence making available sites for PKA that were blocked in our preparations. However, we previously were unable to find any phosphorylated sites in NF-L isolated from rat brain, although there could be phosphorylated sites in regions of NF-L that were not covered by our mass spectrometric sequencing, such as the glutamic acid-rich region (Figure 3, *32*). Finally, a third possibility is that there is a structural/conformational heterogeneity in the neurofilament polypeptides such that few, if any, species are phosphorylated by PKA at all the putative sites, particularly in coassembled NF-L and NF-M. This is certainly borne out by our observation that nonphosphorylated forms of peptides representing all of the sites identified were present in the tryptic digests.

Although serines 2 and 55 are the only sites to have been identified previously (*1, 19*), other phosphopeptides derived from the head region of NF-L (referred to as L1−L3) have been found, but the precise sites have not been identified (*54*). The fact that in our previous (*32*) and this study we have not found these or any other sites to be phosphorylated in NF-L isolated from adult rat brain is consistent with the notion that phosphorylation of neurofilaments is a dynamic process. Our preparation of neurofilaments from brain preferentially selects for assembled neurofilaments, and if phosphorylation is a determinant of unassembled or partially assembled neurofilaments, then it may not be surprising that we did not find any phosphorylated sites. Our finding that serines 43 and 55 were only phosphorylated in vitro by PKA in the recombinant NF-L fusion proteins but not in the mixture of renatured NF-L plus NF-M implies that these two sites may not be accessible to PKA when NF-L is in a complex with NF-M, or that the GST domain prevents NF-L self-assembly, sterically hindering access by PKA to these two sites. On the other hand, serines 2, 41, and 49 in the head domain and serine 435 in the tail domain were all phosphorylated in the NF-L plus NF-M mixture. These observations indicate that further studies of the accessibility and physiological consequences of phosphorylation of these sites in monomeric NF-L and in NF-L in assembled neurofilaments or intermediate assembly complexes deserve further attention both in vitro and in vivo. The additional effects of NF-H coassembly on accessibility of phosphorylation sites is a further aspect that deserves future attention.

Serine 473, a known phosphorylation site both in vitro and in vivo (*31*), is located in the glutamic acid-rich region which was not covered by sequencing. It therefore remains unclear whether this is an in vivo PKA site, but it is not located in a consensus sequence for PKA phosphorylation.

NF-M is multiply phosphorylated in vivo at sites located in the head domain, and PKA has been shown to phosphorylate in vitro some of these sites located in the phosphopeptides referred to as M1−M6 and M8 (*20*). Several head domain sites have been identified (*21*) and may correspond to some of the M peptides, but this is not yet established. In rat brain NF-M, two sites in the amino-terminal head domain were identified (Table 3); serine 46 has been previously reported in mouse (*21*), but serine 1 is a novel PKA site. 51% of the sequence was covered by mass spectrometric sequencing. Sihag and co-workers (*21*) have shown that serines 23, 28, 32, and 41 are in vitro PKA phosphorylation sites in mouse NF-M; serine 23 in our sample was not covered by mass spectrometric sequencing, and although serines 28, 32, and 41 were all covered, none were found to be phosphorylated. However, since both phosphorylated and nonphosphorylated forms of the peptides comprising serine 1 and serine 46 were detected, it suggests that phosphorylation was incomplete. Again the formation of assembly

complexes between the subunits while in the phosphorylation buffer is a likely explanation for these sites not being phosphorylated. We found four phosphorylation sites in the carboxyl-terminal tail domain: serines 603, 608, 666, and 766, which we previously identified in NF-M isolated from rat brain (*32*). Serines 603, 608, and 666 have also been identified by comparison of NF-M peptides from in vitro ^{32}P-labeled cytoskeletal preparations with those from metabolically labeled dorsal root ganglia (*28*). In addition, Yang et al. (*30*) showed that serines 603 and 666 are phosphorylated by GSK-3α in vitro.

Serine and threonine residues commonly phosphorylated by PKA are flanked by basic amino acid residues on the amino and carboxyl sides (*55*), but the sequence R(R/K)X-(S/T) is generally accepted as a PKA consensus motif (*56*). In addition, a hydrophobic residue is often found after serine residues targeted by PKA (*57*). The head domains of both NF-L and NF-M contain multiple serine residues interspersed with arginine and arginine-arginine dipeptides and followed by the hydrophobic residues phenylalanine, tyrosine, leucine, and valine. With the exception of serine 55, the sites identified here in both NF-L and NF-M are not located within a consensus sequence for PKA. However, all of the sites are followed by a hydrophobic residue, and all except serine 2 in NF-L and serine 1 in NF-M are flanked by arginine residues in close proximity on either or both amino and carboxyl sides. Giasson et al. (*19*) showed that in dorsal root ganglion cultures the in vivo phosphorylation of serine 2 in NF-L increased under conditions of PKA activation. PKA also phosphorylated this site in vitro (*19*). Therefore, serine 2 not only is an in vivo site but also is targeted by PKA both in vivo and in vitro, yet it does not exist in a classical PKA consensus sequence. It was suggested by the authors of this publication that serine 2 may be in a context where proximal basic amino acids from a noncontiguous sequence specify phosphorylation (*58*). Although serine 2 is not flanked by basic residues, it is followed by a phenylalanine residue and is in close proximity to a tyrosine residue (S-^{2}**S**-F-S-Y). Similarly, a tyrosine residue follows serine 1 of NF-M. Furthermore, serines 23, 28, 32, 41, and 46 in the head domain of NF-M have been identified as PKA phosphorylation sites in vitro but are not located in classical PKA consensus sequences (*21*) although they are consistent with the less stringent requirements for PKA sites, as discussed above. Clearly, the accepted classical consensus sequence for PKA is not an absolute requirement for phosphorylation, and it is also known that higher orders of structure can influence phosphorylation (*57*).

Given the number of roles that phosphorylation appears to play in neurofilament behavior, the identification of novel phosphorylation sites is important for the elucidation of the complex regulation of neurofilament function, both in normal neurones and in disease states characterized by neurofibrillary pathology. The sensitivity of this technique, coupled with the ease and speed of sample preparation and analysis, should enable neurofilaments and other proteins from a variety of sources to be analyzed in yet greater detail for posttranslational modifications. Our future studies will now particularly address the identification of phosphorylation sites in neurones subjected to various treatments to promote changes in the extent of phosphorylation in vivo and the establishment of the physiological roles of identified sites.

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