Characterization of the Phosphorylation Sites of Human High Molecular Weight Neurofilament Protein by Electrospray Ionization Tandem Mass Spectrometry and Database Searching

Howard Jaffe,*,‡ Veeranna,§ K. T. Shetty,|| and Harish C. Pant§

Protein/Peptide Sequencing Facility and Laboratory of Neurochemistry, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892, and National Institute of Mental Health and Neurosciences, Bangalore, India

Received November 10, 1997

ABSTRACT: Hyperphosphorylated high molecular weight neurofilament protein (NF-H) exhibits extensive phosphorylation on lysine-serine-proline (KSP) repeats in the C-terminal domain of the molecule. Specific phosphorylation sites in human NF-H were identified by proteolytic digestion and analysis of the resulting digests by a combination of microbore liquid chromatography, electrospray ionization tandem (MS/MS) ion trap mass spectrometry, and database searching. The computer programs utilized (PEPSEARCH and SEQUEST) are capable of identifying peptides and phosphorylation sites from uninterpreted MS/MS spectra, and by use of these methods, 27 phosphopeptides and their phosphorylated residues were identified. On the basis of these phosphopeptides, 38 phosphorylation sites in human NF-H were characterized. These include 33 KSP, lysine-threonine-proline (KTP) or arginine-serine-proline (RSP) sites and four unphosphorylated sites, all of which occur in the KSP repeat domain (residues 502−823); and one threonine phosphorylation site observed in a KVPTPEK motif. Six KSP sites were not characterized because of the failure to isolate and identify corresponding phosphopeptides. Heterogeneity in serine and threonine phosphorylation was observed at three sites or deduced to occur at three sites on the basis of enzyme specificity. As a result of the phosphorylated motifs identified (KSPAKEE, KSPVKEE, KS/TPEKAK, KSPEKEE, KSPVKAE, KSPAEAK, KSPPEAK, KSPEAKT, KSPAEVK, and KVPTPEK), human NF-H tail domain is postulated to be a substrate of proline-directed kinases. The threonine-phosphorylated KVPTPEK motif suggested the existence of a novel proline-directed kinase.

The high molecular weight neurofilament protein (NF-H)[1] is among the most hyperphosphorylated of known proteins (*1*). In mature myelinated axons, most of the phosphorylation in NF-H occurs at Lys-Ser-Pro (KSP) repeats in the carboxy-terminal tail domain (*2*−*4*). Phosphorylation of this domain is reported to affect interaction between NF-H and microtubules (*5*−*9*), determine axonal caliber (*8, 9*), protect NF-H from proteolysis (*10, 11*), and contribute to calcium buffering in the axonal compartment (*12*). Phosphorylation may also regulate the distribution of neurofilaments between stationary and mobile phases in the axon (*13*). Aberrant hyperphosphorylation of similar sites in perikaryal NF-H has been shown to be a common feature of many neurological diseases, such as Alzheimer's (*14, 15*), Parkinson's disease (*16, 17*), and amyotrophic lateral sclerosis (*18*−*20*).

Identification of phosphorylated motifs in proteins can provide information on the endogenous kinases that operate on the protein *in vivo.* Identification of phosphorylated and unphosphorylated epitopes in human NF-H has previously been reported utilizing methods involving monoclonal antibodies (*21*). However, these methods could not precisely determine the number of phosphorylated residues in a given protein, nor could they distinguish between the phosphorylation of specific S/T residues in KSP or KTP repeats or in other motifs. It is therefore only by biophysical methods, such as protein sequencing by automated Edman degradation (*22, 23*) or mass spectrometric sequencing (*24*), that the number and specific phosphorylation sites in NF-H can unambiguously be determined.

Mass spectroscopy is finding increasing use in the characterization of protein posttranslational modifications, such as phosphorylation. The highly phosphorylated NF-H represents a challenge for characterization of its phosphorylation sites by mass spectrometry. Recently, the use of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was described in the

* To whom correspondence should be addressed: Fax (301) 496-1339; E-mail jaffe@codon.nih.gov.

‡ LNC−NINDS Protein/Peptide Sequencing Facility.

§ Laboratory of Neurochemistry.

|| National Institute of Mental Health and Neurosciences.

[1] Abbreviations: ESI/MS/MS, electrospray ionization tandem mass spectrometry; NF-H, NF-M, and NF-L, high, medium, and low molecular weight neurofilament proteins, respectively; LC/MS/MS, liquid chromatography tandem mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; IMAC, immobilized metal affinity chromatography; ΔC_n, the difference in normalized correlation score between the top-scoring sequence and the next highest scoring sequence in a PEPSEARCH or SEQUEST search output; P_i, inorganic phosphate; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; CH_3CN, acetonitrile; PrOH, propanol; HOAc, acetic acid; TFA, trifluoroacetic acid.

S0006-2960(97)02518-X CCC: $15.00
Published on Web 02/27/1998

characterization of chicken NF-M (*25*) and nanoelectrospray mass spectrometry in the characterization of the phosphorylation sites of rat low (NF-L) and medium (NF-M) molecular weight neurofilament proteins (*24*). In the latter report, phosphopeptides were characterized by techniques developed by Mann and co-workers (*26*) for the mass spectrometric sequencing of proteins immobilized in a polyacrylamide gel matrix. These techniques require some interpretation of MS/MS spectra to obtain peptide tags (a short stretch of sequence information) prior to database searching and identification of the peptide and its phosphorylation sites *(27, 28*). Although successful in the characterization of the phosphorylation sites of rat NF-M from the minute amounts of protein available in a gel slice, characterization of phosphorylation sites of rat high molecular weight neurofilament protein (rat NF-H) was not described by these authors.

We previously reported on the identification of some of the endogenously phosphorylated KSP sites of rat NF-H by a combination of selective chemical and proteolytic cleavage procedures, P_i determination, microsequencing, and mass spectrometric analysis (*4*). In this paper we describe the mass spectrometric analysis of the endogenous phosphorylation sites of human high molecular weight neurofilament protein. The availability of NF-H in our laboratory as a column-purified lyophilized protein allowed the utilization of a standard solution digestion and electrospray LC/MS/MS procedure. Identification of peptides was accomplished by use of the PEPSEARCH or the similar SEQUEST program described by Yates and co-workers (*29, 30*). These programs are capable not only of correctly identifying peptides in a protein sequence but also of unequivocally identifying precise phosphorylation sites by computer analysis of MS/MS spectra (*31*). This is accomplished without prior interpretation of the MS/MS spectra to determine a sequence tag. To our knowledge, this is the first extensive characterization of the phosphorylation sites in any *human* neurofilament protein.

MATERIALS AND METHODS

Human neurofilament preparations were obtained from human autopsy brain tissue as described (*32, 33*). Human NF-H was purified from a neurofilament preparation as described (*34*). Human NF-H (50 μg) was dried in a Speed Vac (Savant) and taken up and heated in 25 μL of 8 M urea/0.4 M NH_4HCO_3 at 50 °C to effect solubilization and denaturation of the protein essentially according to the method of Stone and Williams (*35*) but without reduction and alkylation. The resulting solution was diluted with 75 μL of H_2O to 2 M urea/0.1 M NH_4HCO_3 prior to digestion overnight with 3 μg of modified trypsin (Promega) at 37 °C or diluted with 175 μL of H_2O to 1 M urea/0.05 M NH_4HCO_3 prior to digestion overnight with 3 μg of endoproteinase Glu-C (Boehringer Mannheim) at ambient temperature. The resulting digests ($^1/_3$ of each) were desalted online by use of a peptide trap cartridge (*36*) prior to separation by microbore RP-HPLC on a 1.0 × 150 mm Monitor C18 column (Column Engineering) and prefilter eluted at 50 μL/min at 40 °C utilizing (A) a two-step gradient of 2−10% solvent B over 20 min followed by 10−65% solvent B over 20 min (2−10−65 gradient), (B) a two-step gradient of 2−15% solvent B over 20 min followed by 15−65% solvent B over 20 min (2−15−65 gradient), or (C) a one-step linear gradient of 2−65% solvent B over 40 min (2−65 gradient) on a Magic 2002 model microbore HPLC (Michrom Bioresources) equipped with a Model 718 refrigerated autosampler (Alcott). The trypsin digest was analyzed twice by the 2−10−65 gradient. Solvent A was 10/10/980/1/0.2 CH_3CN/1-PrOH/H_2O/HOAc/TFA (v/v/v/v/v), and solvent B was 700/200/100/0.9/0.2 CH_3CN/1-PrOH/H_2O/HOAc/TFA (v/v/v/v/v, *37*). Column effluent was monitored at 215 nm.

The HPLC system was coupled to a Model LCQ mass spectrometer (Finnigan) equipped with an electrospray interface (ESI). The mass spectrometer was operated in the "triple play" mode in which the instrument was set up to automatically acquire (A) a full scan, (B) a ZoomScan (higher resolution, lower mass range scan) of the $(M + nH)^{n+}$ ion above a preset threshold and (C) a tandem MS/MS spectrum (relative collision energy = 35%) from that ion. In this way the three mass spectra A−C are automatically acquired from ions resulting from all major peptide peaks eluting from the HPLC column. Source conditions were as follows: capillary temperature, 220 °C; sheath gas flow, 80 units; auxiliary gas flow, 20 units; ESI spray voltage, 4.2 kV. MS data were acquired on a Gateway 2000 computer (Gateway) and analyzed using the BioExplore software package (Finnigan) or converted to Unix format for analysis on an AlphaStation 200 (Digital) utilizing the Bioworks software package (Finnigan). MS/MS spectra ascertained to be from the same precursor ion were added. HPLC operation and MS calibration were checked daily by injection of synthetic peptides, substance P, or kassinin (Peninsula). Uninterpreted MS/MS spectra were searched utilizing the PEPSEARCH (Finnigan) or SEQUEST (version B22 or C1, by J. Eng and J. Yates, Department of Molecular Biotechnology, University of Washington, Box 357730, Seattle, WA 98195-7730) programs against a database constructed from the published human NF-H sequence (*3*). Search parameters were set to reflect the proteolytic enzyme utilized and to consider possible phosphorylation (+80) at all serine, threonine, or tyrosine residues. Fragment ions were interpreted and labeled utilizing the PEPMATCH program (Finnigan).

RESULTS

Human NF-H was purified from brain, by previously described (*32*−*34*) column chromatographic procedures (Figure 1.). Human NF-H was subjected to proteolytic digestion with trypsin or endoproteinase Glu-C and the resulting digests analyzed by LC/MS/MS. Separation of a tryptic digest of human NF-H utilizing a two-step gradient (2−10−65 gradient) designed to provide for isolation and increased resolution of polar phosphopeptides is shown in Figure 2. As expected, polar NF-H phosphopeptides were found to elute during or shortly after the shallow first step of the gradient. Phosphopeptides were identified by examination of their positive mode MS/MS spectra for a dominant ion resulting from the neutral loss of 98 (H_3PO_4) for a singly charged, 49 (H_3PO_4/2) for a doubly charged, 32.7 (H_3PO_4/3) for a triply charged, or 24.5 (H_3PO_4/4) for a quadruply charged ion for each phosphoserine or phosphothreonine residue in the peptide. Upon loss of H_3PO_4, the original serine or threonine is converted to a dehydroalanine or dehydroamino-2-butyric acid residue (*38, 39*).

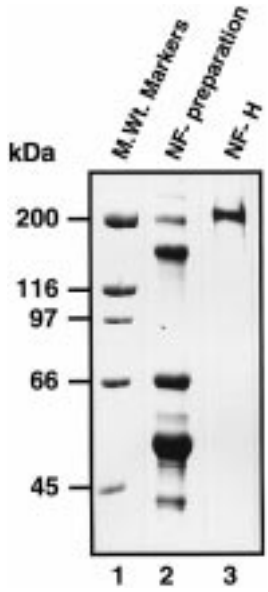

FIGURE 1: SDS−PAGE (10% acrylamide, silver staining) analysis of isolation and purification of human NF-H. Lane 1, molecular mass markers; lane 2, human neurofilament preparation; lane 3, human NF-H.

As seen, for example, in Figure 3, the tandem MS/MS spectrum from a triply charged ion at *m*/*z* 803.4 eluting at 6.1 min (Figure 2) is dominated by three strong ions at *m*/*z* 771.0, 738.3, and 705.7 (Figure 3C). These ions are seen to be consistent with the loss of $1-3 \times 32.7$ (H_3PO_4/3) from the triply charged parent ion at *m*/*z* 803.4 (Figure 3A), which flags this spectrum as resulting from a triply charged triphosphopeptide. The one-third *m*/*z* unit spacing of the isotope cluster in the ZoomScan high-resolution spectrum (Figure 3B) identified the parent ion as triply charged (*40*).

The uninterpreted MS/MS spectrum (Figure 3C) was searched against a database constructed from the reported human NF-H sequence (*3*) utilizing PEPSEARCH (*41*) or the similar SEQUEST (*29*−*31*) program. Search parameter files were modified to consider possible phosphorylation (+80) at all serine, threonine, or tyrosine residues, and enzyme specificity was set to trypsin. The automated output (Table 1) of the search unequivocally identified (rank = 1, correlation score = 1) the MS/MS spectrum as derived from the triphosphopeptide HT14 (Table 3A): (K)AKS*-PVKEEAKS*PEKAKS*PEK.

The peptide is seen to have resulted from an expected cleavage by trypsin (K or R) and its experimental mass of 2408.7 is seen to be in good agreement with the calculated mass of 2408.4 (Table 3A). The second ranked peptide (Table 1) is seen to display a ΔC_n of 0.349 (the difference in normalized correlation score between the top-scoring sequence and the next highest scoring sequence), which, when greater than 0.1, is indicative of a high degree of confidence in a correct match for the first ranked peptide (*29*).

Similarly, LC/MS/MS analysis of the endoproteinase Glu-C digest of human NF-H resulted in a MS/MS spectrum (Figure 4) from a triply charged ion at *m*/*z* 725.6 derived from a peptide eluting at 23.7 min (chromatogram not shown). The spectrum is seen to be dominated by a strong ion at *m*/*z* 692.9. This ion is seen to be consistent with the loss of 1×32.7 (H_3PO_4/3) from the triply charged parent ion at *m*/*z* 725.6, which flags this spectrum as resulting from a triply charged monophosphopeptide. The one-third *m*/*z* unit spacing of the isotope cluster in the ZoomScan high-resolution spectrum again identified the parent ion as triply charged (not shown). Database searching (Table 2) as above unequivocally identified (rank = 1, correlation score = 1, ΔC_n of second ranked peptide = 0.372) the MS/MS spectrum as derived from the monophosphopeptide HE5 (Table 3B): (E)AEDKKKVPT*PEKEAPAKVE. The peptide is seen to have resulted from an expected cleavage of endoproteinase Glu-C (E), and its experimental mass of 2173.8 is seen to be in good agreement with the calculated mass of 2174.2 (Table 3B).

Using similar methodology to that employed in the analysis of phosphopeptides HT14 and HE5, phosphopeptides HT3−HT6, HT8−HT14, HT16, HT18−HT20, HE4, HE6, and HE7 (Table 3) were identified, where HT and HE denote trypsin or endoproteinase Glu-C phosphopeptides, respectively. In all these phosphopeptides, the number of phosphates found by observation of the number of dominant $-H_3PO_4$ ions in the MS/MS spectra equaled the number of serines or threonines in the first ranked peptide of the program output. Assignment of phosphorylation sites was straightforward and based simply on selection of the first ranked peptide (correlation score = 1) provided that the ΔC_n of the second ranked peptide was greater than 0.1. In all cases the first ranked peptide was consistent with the cleavage expected for the enzyme used. Additional confirmation of the assignment came from good agreement of the experimental and calculated molecular weight and a match of a significant number of the observed and predicted ions. These two factors are criteria used, in part, by the programs in the ranking of the peptides (*29*−*31*, *41*).

In phosphopeptides HT1, HT2, HT7, HT15, HT17, and HE1−HE3 (Table 3), the number of phosphates found by observation of the number of dominant $-H_3PO_4$ ions in the MS/MS spectra was less than the number of serines or threonines in the first ranked peptide in the program output. Assignment of phosphorylation sites was similarly based on selection of the first ranked peptide, which included assignment of phosphorylated residues (*31*). As seen, for example in Table 4, for the first ranked human NF-H diphosphopeptide HE1, (E)AKS*PEKAKS*PEKAKTLDVKSPE, two of the four potential phosphorylated residues have been assigned by the SEQUEST program to residues 746 and 752. The second ranked peptide, (E)AKS*PEKAKSPEKAKT*-LDVKSPE, which is seen to be the same peptide but with phosphorylated residues assigned to residues 746 and 758, displays a ΔC_n of 0.365 and was thus rejected.

In the case of diphosphopeptide HT1, EEAKSPAEAKS*-PEKEEAKSPAEVKS*PEKAK, assignment by SEQUEST of phosphorylated residues was confirmed by examination of the fragment ions of its MS/MS spectrum. In addition to the two dominant $-H_3PO_4$ ions at *m*/*z* 819.2 and 794.6 that characterize the spectrum as resulting from a quadruply charged diphosphopeptide, b_5^{+1}, b_7^{+1}, b_8^{+1}, bo_5^{+1}, a_7^{+1}, and a_{10}^{+1} ions consistent with a nonphosphorylated serine at residue 526 (residue 5 in the peptide) were observed. Having established that residue 526 in this peptide is a nonphosphorylated serine, the observation of a_{11}^{+1}, a_{17}^{+2}, a_{18}^{+2}, bo_{12}^{+2}, b_{16}^{+2}, and p_{12}^{+1} ions that exhibit mass shifts of +80 Da (+1) or +40 Da (+2) establishes residue 532 (residue 11 in the

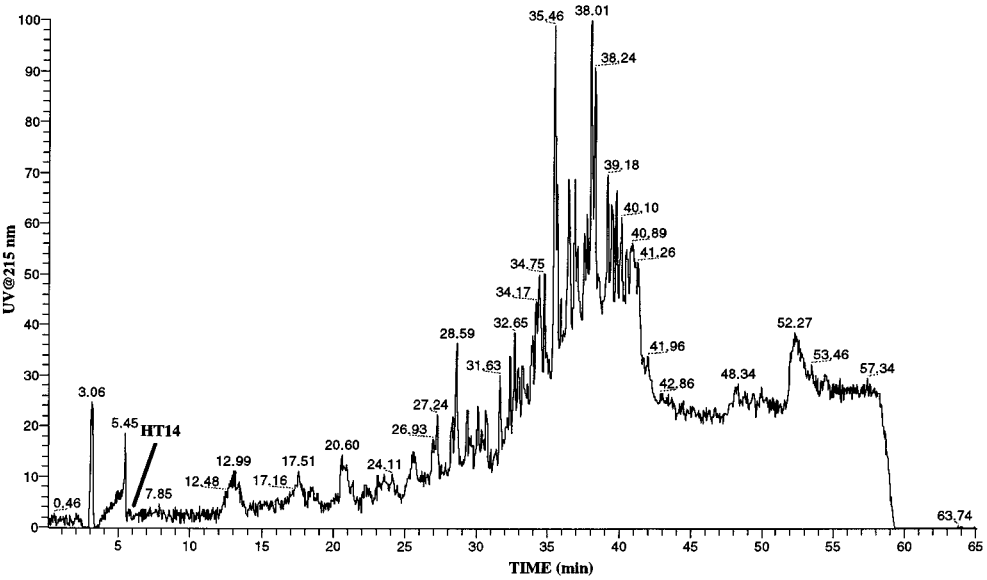

FIGURE 2: Microbore RP-HPLC analysis of trypsin digest of 17 μg of human high molecular weight neurofilament protein by two step 2−10−65 gradient (see Materials and Methods). UV absorbance was monitored at 215 nm. Phosphopeptides eluted between 5.8 and 25.5 min. Phosphopeptide HT14 (Table 3A) eluting at 6.1 min is indicated.

peptide) as a phosphoserine. Similarly, the observation of yo_7^{+1}, y_8^{+1}, y_{10}^{+1}, and $y^*_{10}{}^{+3}$ ions that exhibit mass shifts of +80 Da (+1) or +26.7 Da (+3) establishes residue 546 (residue 25 in the peptide) as a phosphoserine. Having established residue 546 as a phosphoserine, the observation of y_{14}^{+2}, y_{15}^{+3}, y_{16}^{+3}, and y_{17}^{+2} ions that exhibit mass shifts of +40 Da (+2) or +26.7 Da (+3) resulting from the phosphoserine at residue 546, but no additional shifts from residue 540 (residue 19 in the peptide), establishes residue 540 as a nonphosphorylated serine. In addition, a_{15}^{+3}, a_{16}^{+2}, a_{17}^{+4}, a_{24}^{+3}, a_{27}^{+3}, b_{17}^{+2}, b_{15}^{+3}, b_{30}^{+3}, y_7^{+1}, yo_9^{+1}, y_{10}^{+1}, y_{16}^{+3}, y_{26}^{+3}, $y^*_{26}{}^{+2}$, y_{10}^{+4}, p_{11}^{+1}, and p_{17}^{+3} fragment ions from the series of ions resulting from the loss of 1 or 2 H_3PO_4 (phosphoserine converted to dehydroalanine) and consistent with phosphorylation at residues 532 and 546 were also observed. Only ions with at least 4% of the intensity of the base peak ion at *m*/*z* 819.2 (31 937 counts) were considered.[2]

There were MS/MS data for several phosphopeptides for which the second and sometimes also the third peptide in the SEQUEST or PEPSEARCH outputs exhibited ΔC_n values of <0.1. Those peptides usually differed only in the arrangement of their phosphorylated residues and since, for those peptides, the program could not distinguish with a high degree of confidence (ΔC_n < 0.1) among the first, second, and sometimes third ranked peptides listed in the program output, the data for those peptides were accordingly rejected (data not shown).

DISCUSSION

Previous reports on the characterization of phosphopeptides in digests by mass spectrometry have utilized an immobilized metal affinity chromatography (IMAC) step both in-line prior to ESI/MS (*42*) and off-line prior to nanoelectrospray mass spectrometry (*24*) to isolate phosphopeptides from digests. This step was inserted in order to avoid coelution of phosphopeptides with other peptides in the digest, which could result in ion suppression of phosphopeptides (*24*). In the analysis of NF-H proteins, we have taken advantage of the fact that most of the expected phosphopeptides resulting from trypsin or endoproteinase Glu-C proteolytic cleavage of the hyperphosphorylated tail region would be expected to be polar because of the presence of a high percentage of polar residues, such as S, S*, T, T*, K, D, and E. These peptides would elute early in the low percentage solvent B part of a reversed-phase gradient, where solvent B consists of mainly organic modifier (Materials and Methods). We thus eliminated the IMAC step and employed two-step gradients, in which the first step consisted of a shallow linear gradient of 2−10% or 2−15% solvent B, followed in each case by a second step consisting of a steep linear gradient to 65% solvent B (2−10−65 or 2−15−65 gradients). The two-step gradients were designed to isolate polar phosphopeptides during the first step and to provide for their increased resolution. Also employed for comparison purposes was a standard one-step linear gradient of 2−65% solvent B (2−65 gradient).

Both two-step gradients were successful in that phosphopeptides did elute during the first step of the gradient or shortly thereafter, and most of the other peptides eluted during the second step. The 2−10−65 gradient, with the shallowest 2−10% first step, resulted in the detection and characterization of 13 human NF-H tryptic phosphopeptides, as compared to 12 for the 2−15−65 gradient and 5 for the 2−65 gradient. Interestingly, some different phosphopeptides were characterized in all three gradients (Table 3), possibly reflecting effects of the different chromatographic conditions on the peptide resolution, ionization, and detection by the mass spectrometer. In light of the similar number of phosphopeptides characterized by either of the two-step gradients, either is recommended for LC/MS/MS analysis of polar phosphopeptides in digests.

MS/MS spectra were flagged as derived from a phosphopeptide by the presence of a major ion or ions corresponding to the neutral loss of H_3PO_4 (*43*) for each phosphoserine or

[2] Fragment ions >1000 cts in LCQ MS/MS spectra are considered chemically significant (A. P. Land, Finnigan MAT, San Jose, CA, personal communication).

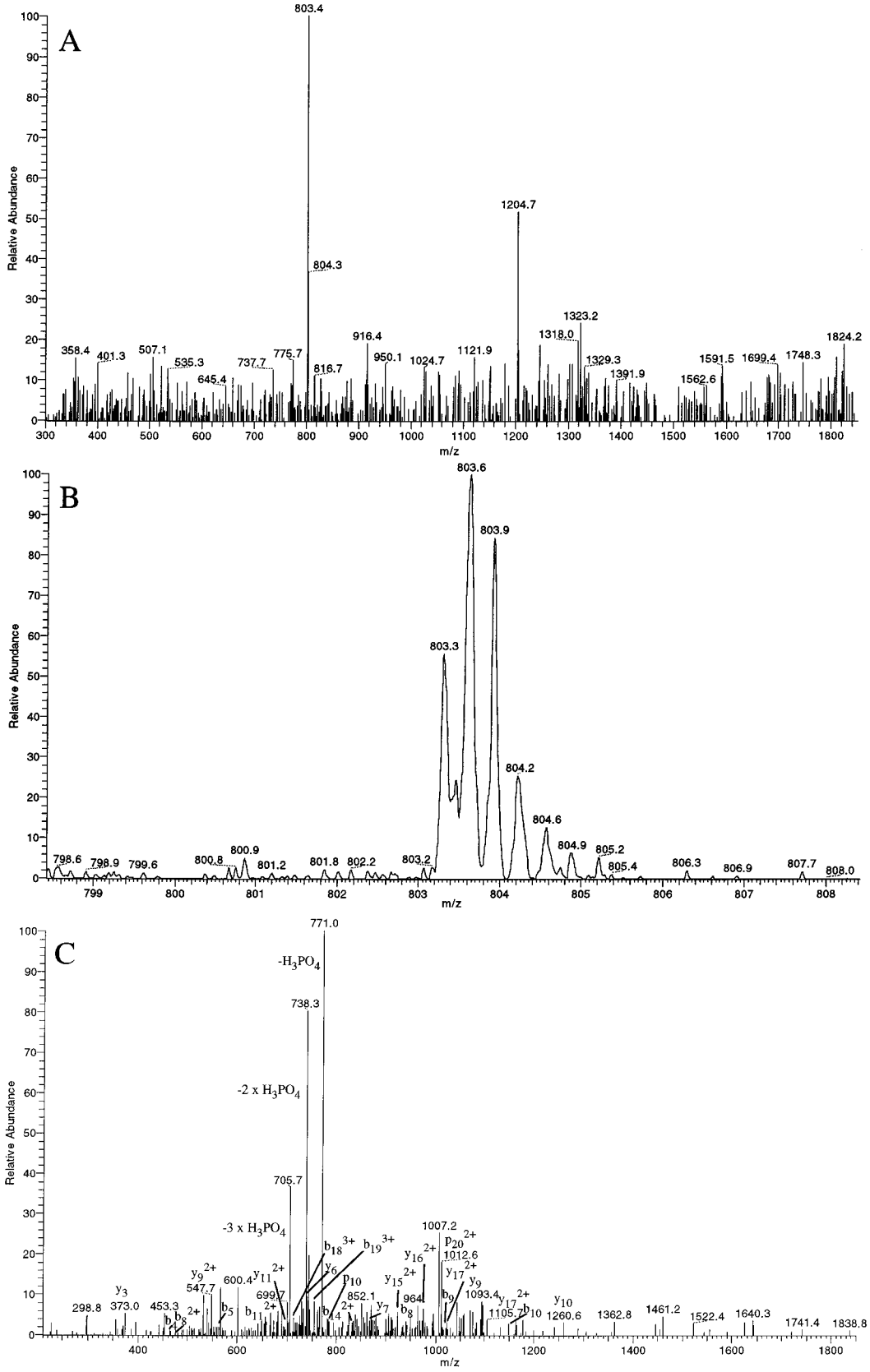

FIGURE 3: "Triple play" analysis (see Materials and Methods) of the triply charged ion at *m*/*z* 803.4 from the human NF-H tryptic triphosphopeptide HT14 (Table 3A), AKS*PVKEEAKS*PEKAKS*PEK, eluting at 6.1 min. (A) Full-scale scan MS spectrum of the triply charged ion at *m*/*z* 803.4. (B) Zoom scan of the triply charged ion at *m*/*z* 803.4. Smoothing algorithm (7 points) applied. The one-third *m*/*z* unit spacing of the isotope cluster in the ZoomScan high-resolution spectrum identifies the parent ion as triply charged. (C) Full-scan MS/MS spectrum of the triply charged ion at *m*/*z* 803.4. The spectrum is dominated by three ions at *m*/*z* 771.0, 738.3, and 705.7, consistent with the loss of $1-3 \times 32.7$ (H_3PO_4/3) from the triply charged parent ion at *m*/*z* 803.4, which flags this spectrum as resulting from a triply charged triphosphopeptide. Observed a,b, b*, bo, y, y*, yo and p ions (*52*−*54*) consistent with those predicted for HT14 and at least above a threshold of 4% of the base peak counts (140 681 counts) are labeled.

Table 1: Result Output of PEPSEARCH Search against a Database Constructed from the Reported Human NF-H Sequence[a] of the MS/MS Spectrum Shown in Figure 3C[b]

no.	rank	C_n	ΔC_n	protein	mult	ions	subsequence
1	1	1.000	0.0000	hNF-H[c]		34/76	(K)AKS*PVKEEAKS*PEKAKS*PEK[d]
2	2	0.651	0.349	hNF-H		26/76	(K)KAKS*PVKEEAKS*PEKAKS*PE
3	3	0.649	0.351	hNF-H	+1	24/76	(S)PEKEEAKS*PAEVKS*PEKAKS*
4	4	0.600	0.400	hNF-H	+1	23/76	(E)AKS*PEKAKS*PVKEEAKS*PEK
5	5	0.585	0.415	hNF-H	+2	17/76	(S)PEKAKS*PVKEEAKS*PEKAKS*
6	6	0.493	0.507	hNF-H	+1	28/76	(E)AKS*PEKEEAKS*PAEVKS*PEK
7	7	0.459	0.541	hNF-H		23/76	(A)KS*PVKEEAKS*PAEAKS*PEKE
8	8	0.431	0.569	hNF-H		26/76	(A)KSPEKAKS*PEKAKT*LDVKS*P
9	9	0.407	0.593	hNF-H		24/76	(K)SPEKAKS*PEKAKT*LDVKS*PE
10	10	0.383	0.617	hNF-H	+1	20/76	(K)S*PEKEEAKS*PAEVKS*PEKAK

[a] From Lees et al. (*3*). [b] Table parameters are as previously described (*29, 30*): rank signifies the result of analysis of the sequences using a cross-correlation function; C_n is the normalized score from the cross-correlation function; ΔC_n is the difference between the cross-correlation parameter of the top-scoring sequence and the listed sequence; mult is the number of additional times the subsequence occurs in the protein(s) in the database; ions is the number of ions of the type y, y*, yo, b, b*, bo, or a (*52*−*54*) observed in the MS/MS spectrum versus the number predicted. [c] hNF-H is human high molecular weight neurofilament protein. [d] S* and T* indicate phosphorylated residues; parentheses indicates the amino acid residue immediately preceding the peptide.

Table 2: Result Output of PEPSEARCH Search against a Database Constructed from the Reported Human NF-H Sequence[a] of the MS/MS Spectrum Shown in Figure 4

no.	rank	C_n	ΔC_n	protein	mult	ions	subsequence
1	1	1.000	0.0000	hNF-H		26/72	(E)AEDKKKVPT*PEKEAPAKVE
2	2	0.628	0.372	hNF-H		21/68	(R)QRSELEDRHQADIASYQE
3	3	0.611	0.389	hNF-H		20/84	(K)GGAGGTRSAAGSS*SGFHSWTRT
4	4	0.581	0.419	hNF-H		19/84	(K)GGAGGT*RSAAGSSSGFHSWTRT
5	5	0.533	0.467	hNF-H		18/84	(K)GGAGGTRSAAGS*SSGFHSWTRT
6	6	0.507	0.493	hNF-H		17/68	(K)PKTEAKAKEDDKT*LS*KEP
7	7	0.486	0.514	hNF-H		20/76	(E)AEGGEEETKS*PPAEEAAS*PE
8	8	0.483	0.517	hNF-H		17/84	(K)GGAGGTRS*AAGSSSGFHSWTRT
9	9	0.433	0.567	hNF-H		26/68	(D)DKTLS*KEPS*KPKAEKAEK
10	10	0.423	0.577	hNF-H		23/72	(I)DKVRQLEAHNRS*LEGEAAA

[a] See footnotes in Table 1.

phosphothreonine present. In almost all cases these ions were the most abundant ions in the MS/MS spectra. This striking feature was observed even for tetraphosphopeptides, such as HT4, HT8, HT12, and HT13, where four $-H_3PO_4$ ions were clearly evident (Figure 5). As might be expected however, there was a trend toward decreasing abundance with increasing number of H_3PO_4 lost and in some cases the ion corresponding to loss of $4 \times H_3PO_4$ was no longer a major ion. Identification of peptides was accomplished by use of the PEPSEARCH or the similar SEQUEST programs, which were searched against a database constructed from the published sequence of human NF-H (*3*). These programs are capable not only of correctly identifying peptides in the NF-H sequence (*44*) but also of unequivocally identifying the precise phosphorylation site(s) by analysis of their uninterpreted MS/MS spectra (*29*−*31, 41*). This was first shown by Yates and co-workers (*31*) for the tryptic α-casein diphosphopeptide DIGS*ES*TEDQAMEDIK, in which the phosphorylation sites were correctly identified as a first ranked peptide by SEQUEST upon a search of its MS/MS spectrum against the entire OWL database (*31*).

Unequivocal identification of peptides and phosphorylation sites was subject to ΔC_n constraints described above. There were several obvious phosphopeptide ions observed, whose phosphorylation sites could not be unequivocally determined by SEQUEST or PEPSEARCH. These data were rejected and not included in Table 3. For all the 21 doubly and triply charged peptides listed in Table 3, except for a cursory review to ensure that the spectra were of high quality and contained sufficient fragment ions for analysis by SEQUEST, no further analysis or manual interpretation of MS/MS or MS/MS/MS spectra (*38, 39*) was necessary for confirmation of the NF-H phosphorylation sites. However, in the case of the remaining six large (>3000 Da), quadruply charged phosphopeptides, HT1, HT4, HT8, HT12, HT13, and HE4, where it is possible that insufficient fragmentation had occurred to unequivocally validate computer assignments, the spectra were scrutinized to ensure that they contained sufficient fragment ions for analysis by SEQUEST. In five of the peptides, HT4, HT8, HT12, HT13, and HE4, the number of phosphates determined by the dominant $-H_3PO_4$ ions in the MS/MS spectra equaled the number of serines or threonines in the peptide and, in addition, assignment of phosphorylation sites for these peptides was possible by mass alone. Only in the case of HT1, in which two out of four potentially phosphorylated residues were assigned by SEQUEST, was confirmation by manual examination of fragment ions deemed prudent, as described above.

It should be noted that interpretation of MS/MS/MS spectra, which normally can be used to confirm the analysis of MS/MS spectra (*38, 39*), is extremely complicated for the multiphosphorylated peptides encountered in digests of the hyperphosphorylated NF-H proteins. The precursor ion (major ion in the MS/MS spectrum) for a MS/MS/MS spectrum of a multiphosphorylated NF-H peptide would be a species in which only one out of two, three, or four serines or threonines has been converted to a dehydroalanine or dehydroamino-2-butyric acid residue. Interpretation of this spectrum could prove to be exceedingly difficult, if not impossible, since it is not known which serine or threonine

Table 3: LC/MS/MS Identification of Phosphopeptides from Human NF-H Trypsin and Endoproteinase Glu-C Digests

peptide	residues	ion	exp mass	calc mass	phosphopeptide sequence
			(A) Human NF-H Trypsin Digest		
HT1[a]	522−551	+4	3370.2	3370.5[b]	(K)EEAKSPAEAKS*PEKEEAKSPAEVKS*PEKAK(S)[c]
HT2	540−549, 574−583, 628−637	+2	1151.0	1151.2	(K)SPAEVKS*PEK(A)
HT3	550−573	+3	2805.3	2806.8	(K)AKS*PAKEEAKS*PPEAKS*PEKEEAK(S)
HT4	556−583	+4	3356.4	3357.3	(K)EEAKS*PPEAKS*PEKEEAKS*PAEVKS*PEK(A)
HT5	590−613	+3	2808.3	2808.8	(K)EEAKS*PAEAKS*PEKAKS*PVKEEAK(S)
HT6	604−627	+3	2777.8	2778.8	(K)AKS*PVKEEAKS*PAEAKS*PVKEEAK(S)
HT7	614−637	+3	2763.9	2763.8	(K)SPAEAKS*PVKEEAKS*PAEVKS*PEK(A)
HT8	652−679	+4	3328.8	3329.3	(K)AKS*PEKEEAKS*PEKAKS*PVKAEAKS*PEK(A)
HT9	666−679, 680−693	+2	1628.6	1629.7	(K)AKS*PVKAEAKS*PEK(A)
HT10	694−707, 708−721, 736−749	+2	1687.4	1687.7	(K)AKS*PVKEEAKS*PEK(A)
HT11	694−717, 708−731	+3	2778.6	2778.8	(K)AKS*PVKEEAKS*PEKAKS*PVKEEAK(S) or(T)
HT12	694−721	+4	3356.8	3357.4	(K)AKS*PVKEEAKS*PEKAKS*PVKEEAKS*PEK(A)
HT13	722−749	+4	3370.8	3371.5	(K)AKS*PVKEEAKT*PEKAKS*PVKEEAKS*PEK(A)
HT14	736−755	+3	2408.7	2408.4	(K)AKS*PVKEEAKS*PEKAKS*PEK(A)
HT15	758−767	+2	1167.0	1167.2	(K)TLDVKS*PEAK(T)
HT16	785−798	+2	1715.6	1715.7	(K)AKS*PVKEEVKS*PEK(A)
HT17	787−798	+2	1436.0	1436.5	(K)SPVKEEVKS*PEK(A)
HT18	813−825	+3	1591.5	1590.8	(K)EIPKKEEVKS*PVK(E)
HT19	813−834	+3	2686.8	2688.0	(K)EIPKKEEVKS*PVKEEEKPQEVK(V)
HT20	817−834	+3	2220.0	2220.4	(K)KEEVKS*PVKEEEKPQEVK(V)
			(B) Human NF-H Endoproteinase Glu-C Digest		
HE1[d]	744−765	+3	2528.1	2528.7	(E)AKS*PEKAKS*PEKAKTLDVKSPE(A)
HE2	755−773	+3	2149.2	2150.4	(E)KAKTLDVKS*PEAKTPAKEE(A)
HE3	774−792	+3	2193.3	2194.4	(E)ARSPADKFPEKAKS*PVKEE(V)
HE4	820−845	+4	3098.8	3099.4	(E)VKS*PVKEEEKPQEVKVKEPPKKAEEE(K)
HE5	897−915	+3	2173.8	2174.2	(E)AEDKKKVPT*PEKEAPAKVE(V)
HE6	897−918	+3	2530.2	2530.8	(E)AEDKKKVPT*PEKEAPAKVEVKE(D)
HE7	899−918	+3	2330.1	2330.6	(E)DKKKVPT*PEKEAPAKVEVKE(D)

[a] Human NF-H tryptic phosphopeptides HT1−HT20 in order of increasing residue number. Phosphopeptides from two analyses by 2−10−65 gradient (Materials and Methods): HT1, HT3−HT9, HT11, HT12, and HT14−HT20. Phosphopeptides from analysis by 2−15−65 gradient: HT2, HT6, HT7, HT9, HT10, HT12-HT16, HT19, and HT20. Phosphopeptides from analysis by 2−65 gradient: HT8, HT11, HT14, HT19, and HT20. [b] Average mass. [c] S* and T* indicate phosphorylated residues; parentheses indicates the amino acid residue immediately preceding or following the peptide. [d] Human NF-H endoproteinase Glu-C phosphopeptides HE1−HE7 in order of increasing residue number. Phosphopeptides from analysis by 2−10−65 gradient (Materials and Methods): HE1−HE3, HE5, and HE7. Phosphopeptides from analysis by 2−15−65 gradient: HE1 and HE3−HE6. Phosphopeptides from analysis by 2−65 gradient: HE1 and HE6.

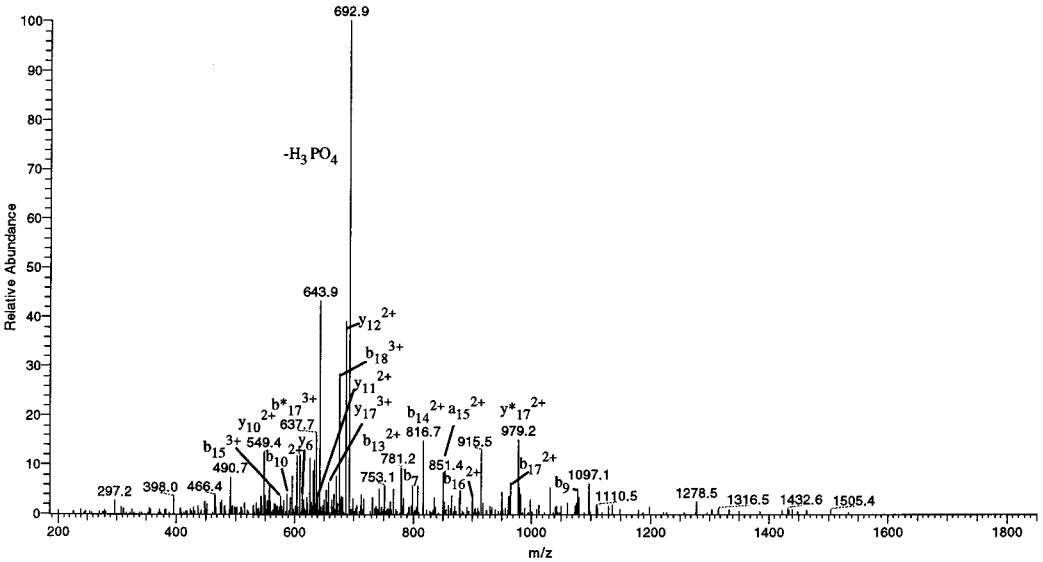

FIGURE 4: Full-scan MS/MS spectrum from "triple play" analysis (see Materials and Methods) of *m/z* 725.6 triply charged ion from the human NF-H endoproteinase Glu-C monophosphopeptide, HE5 (Table 3B), AEDKKKVPT*PEKEAPAKVE, eluting at 23.7 min (chromatogram not shown). The spectrum is dominated by a strong ion at *m/z* 692.9 consistent with the loss of 1 × 32.7 (H_3PO_4/3) from the triply charged parent ion at *m/z* 725.6, which flags this spectrum as resulting from a triply charged monophosphopeptide. Observed a,b, b*, bo, y, y*, yo and p ions (*52*−*54*) consistent with those predicted for HE5 and at least above a threshold of 4% of the base peak counts (2 164 575 counts) are labeled.

was so converted. Moreover, it is also likely that the precursor ion is actually a mixture (in an unknown ratio) of ions with identical mass corresponding to the loss of one H_3PO_4 from each or some of the different phosphorylated residues originally present in the peptide.

By use of the methods described above, 38 phosphorylation sites were characterized in human NF-H (Figure 6), of which 33 were KSP, KTP, or RSP sites, including four sites

Table 4: Result Output of SEQUEST Search against a Database Constructed from the Reported Human NF-H Sequence[a] of the MS/MS Spectrum of the Diphosphopeptide HE1 (Table 3B)

no.	rank	C_n	ΔC_n	protein	mult	ions	subsequence
1	1	1.000	0.0000	hNF-H		30/126	(E)AKS*PEKAKS*PEKAKTLDVKSPE
2	2	0.635	0.365	hNF-H		24/126	(E)AKS*PEKAKSPEKAKT*LDVKSPE
3	3	0.539	0.461	hNF-H		18/126	(E)AKSPEKAKS*PEKAKT*LDVKSPE
4	4	0.439	0.561	hNF-H		22/126	(E)AKS*PEKAKSPEKAKTLDVKS*PE
5	5	0.289	0.711	hNF-H		13/126	(E)KEEAKSPEKAKS*PVKAEAKS*PE
6	6	0.282	0.718	hNF-H		20/126	(E)KEEAKS*PEKAKS*PVKAEAKSPE
7	7	0.245	0.755	hNF-H		14/126	(E)AKSPEKAKSPEKAKT*LDVKS*PE
8	8	0.179	0.821	hNF-H		16/126	(E)AKSPEKAKS*PEKAKTLDVKS*PE
9	9	0.102	0.898	hNF-H		13/126	(E)KEEAKS*PEKAKSPVKAEAKS*PE
10	10	0.014	0.986	hNF-H	+1	19/126	(E)KEEAKS*PAEVKS*PEKAKSPAKE

[a] See footnotes in Table 1.

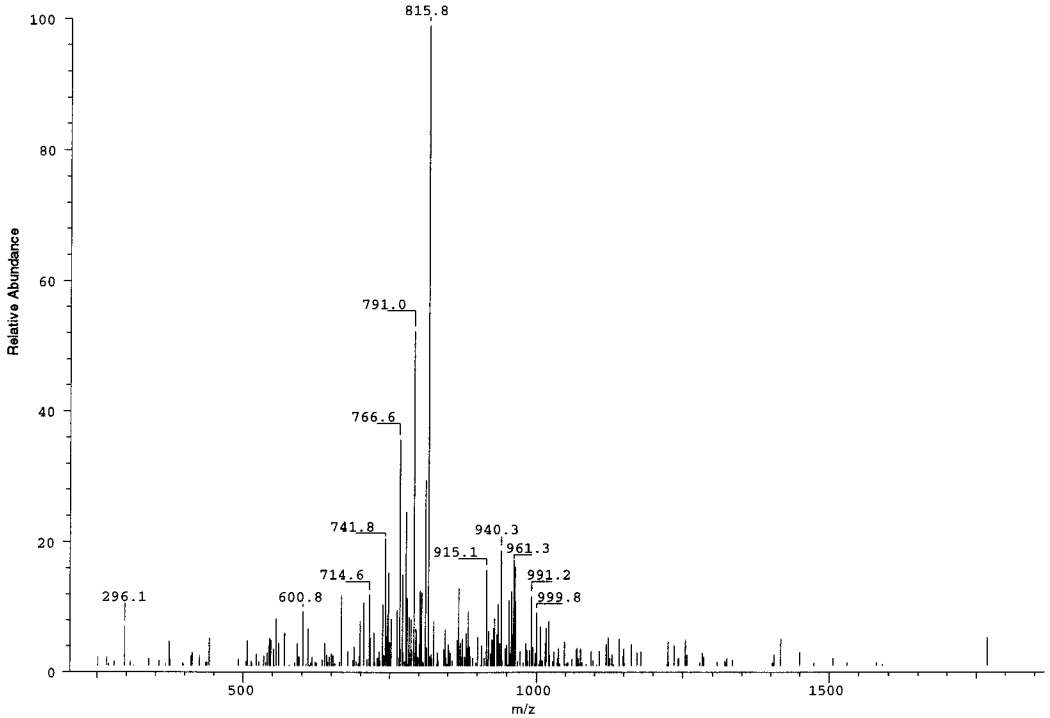

FIGURE 5: Full-scan MS/MS spectrum of a *m*/*z* 840.1 quadruply charged ion from a tryptic tetraphosphopeptide, HT4 (Table 3A), EEAKS*PPEAKS*PEKEEAKS*PAEVKS*PEK, eluting at 19.7 min. The spectrum is dominated by three ions at *m*/*z* 815.8, 791.0, and 766.6, consistent with the loss of $1-3 \times 24.5$ (H_3PO_4/4) from the triply charged parent ion at *m*/*z* 840.1 Also evident is an ion at *m*/*z* 741.8, corresponding to the loss of the 4×24.5 (H_3PO_4/4), which flags this spectrum as resulting from a quadruply charged tetraphosphopeptide.

at residues 526, 540, 768, and 776 (indicated in plain text in Figure 6), which were characterized as unphosphorylated on the basis of phosphopeptides HT1, HE2, and HE3 (Table 3). Also characterized as phosphorylated was a threonine in one PTP site. Included in the total human NF-H KSP sites were sites characterized as a result of four peptides, HT2, HT9, HT10, and HT11 (Table 3A), each of which occurs in two or three locations in the sequence. Since the precise locations from which these peptides were derived are not known, some uncertainty thus exists in the characterization of several of the KSP sites. Of these four peptides, KSP sites in three, HT2, HT10, and HT 11 (in all of their multiple occurrences), are seen to be included in peptides HT1, HT4, HT7, and HT12−HT14. KSP sites in the two occurrences of the remaining peptide, HT9, are only partially included in peptide HT8, leaving some uncertainty in the characterization of two KSP sites on residues 682 and 690 as indicated in Figure 6 as *S*. The 37/43 KSP, RSP, and KTP characterized sites (including residues 682 and 690) cover almost the entire KSP repeat region 502−823, with six KSP sites on residues 503, 518, 586, 640, 648, and 801 remaining uncharacterized (see Figure 6). The failure to characterize the six sites described above can be attributed to several technical reasons, including a failure of enzymatic cleavage to generate the appropriate peptides, a failure of the peptides that were too polar to be retained on the peptide trap, or a failure of the appropriate peptides to form abundant ions upon electrospray ionization. Some of the uncharacterized sites are undoubtedly covered by some of the phosphopeptides whose MS/MS spectral data were rejected as described above. These data might prove useful in the future with improvements in computer programs for the interpretation of MS/MS and MS/MS/MS spectra. An alternate approach might involve synthesis of the first, second, or third ranked phosphopeptides and comparison of spectra to select the correct one. This approach was not attempted in this study. Nevertheless, the results obtained here confirm the high degree of phosphorylation previously reported for the KSP repeat region of NF-H (*4*).

The 37/43 KSP, RSP, and KTP sites characterized in human NF-H are distributed across the C-terminal KSP repeat region (Figure 6) in contrast to the nine sites

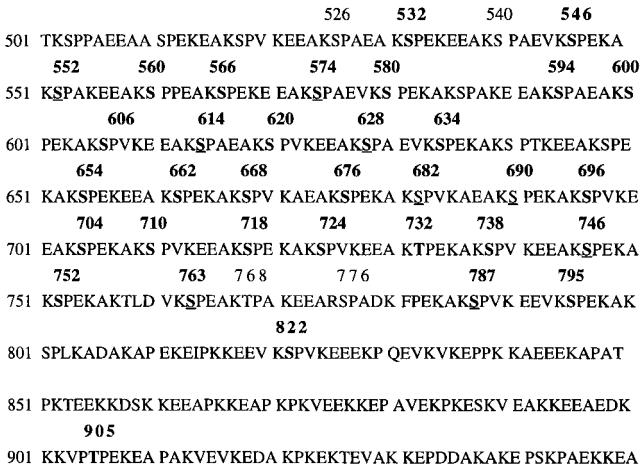

FIGURE 6: Characterization of phosphorylation sites in the tail domain of human high molecular weight neurofilament protein. Phosphorylated residues and residue number are shown in boldface type (**S** or **T**). Phosphorylated residues identified on the basis of a phosphopeptide that occurs more than once are shown as underlined (S or T). Residues that were identified as phosphorylated and nonphosphorylated on the basis of different phosphopeptides or on the basis of enzyme specificity (heterogeneous phosphorylation sites) are shown as boldface underlined (**S** or **T**). Residue numbers characterized as nonphosphorylated are shown in plain text. Amino acid residues were numbered according to Lees et al. (*3*).

previously characterized in rat NF-H, all of which are located on the periphery of the KSP repeat region (*4*). Lack of characterization of any of the rat KSP sites from the core of the KSP repeat region is consistent with the previously described resistance to proteolytic digestion of the rat NF-H, KSP repeat region. This could be due to the regular 6 amino acid spacing of the KSP repeats in rat NF-H which contains a high percentage of negatively charged residues (E, S*). Trypsin has been reported to exhibit considerably reduced activity when acidic residues are present on either side of a potentially susceptible bond.[3] In contrast, human NF-H has a more "open" irregularly alternating 6 and 8 amino acid spacing of its KSP repeats which might account for its susceptibility to proteolytic digestion. Successful proteolytic digestion of the rat NF-H core KSP repeat region utilizing a highly specific proteolytic enzyme, such as trypsin or endoproteinase Glu-C, should facilitate a more complete characterization of the phosphorylation sites using the LC/MS/MS methodology employed for human NF-H. Such experiments are currently underway in our laboratory.

As previously reported for rat NF-M, we also observed heterogeneity in the phosphorylation of human NF-H. Examination of Table 3 and Figure 6 reveals a significant degree of heterogeneity in serine or threonine phosphorylation in human NF-H at residues 614, 763, and 787 and, in addition, in residues 552, 574, and 628, based on the enzyme specificity of trypsin, which would not be expected to have cleaved a KS* bond (*24*). These results are in accord with previous suggestions of neurofilament protein heterogeneity (*1*), but the hydrolytic loss of phosphate (*24*) or loss of phosphate through the action of endogenous phosphatases during isolation and purification cannot be ruled out. Also, as reported for rat NF-M, no phosphorylated sites were characterized from the head domain of human NF-H, which is consistent with the hypothesis that head domain phosphorylation disassembles the neurofilament (*45, 46*)

Characterization of phosphorylated motifs in proteins can provide information on the endogenous kinases that operate on the protein in vivo, information that cannot necessarily be obtained from in vitro studies. In this study we have shown that almost all, except one (see below), of the phosphorylation sites in the tail domain of human NF-H are proline-directed and are located in KS/TP repeats (Figure 6). Thus human NF-H is an in vivo substrate of proline-directed kinases. In the nervous system, these include cyclin-dependent kinases or cdc2-like kinases (*47, 48*), the mitogen-activated protein kinase (MAPK) family (*49*), and glycogen synthase kinase 3 (*50*).

In human NF-H there are 43 K/RS/TP repeats; we have identified 33 phosphorylated S/T residues in these repeats with the following motifs: KSPAKEE, KSPVKEE, KS/TPEKAK, KSPEKEE, KSPVKAE, KSPAEAK, KSPPEAK, KSPEAKT, and KSPAEVK (when arranged in a heptad of amino acid residues). On the basis of kinase consensus sequences, all these residues can be phosphorylated by proline-directed kinases. Consistent with this hypothesis, we have recently shown that active cdk5, either purified from neuronal tissue, or as a complex of expressed cdk5 and its brain-specific activator p35 (or its truncated forms p25 or p21), phosphorylates KSPXK peptides or dephosphorylated NF-H. In fact cdk5 not only phosphorylated dephosphorylated human NF-H but also produced a significant decrease in its electrophoretic mobility on SDS−PAGE gels, suggesting that cdk5 is a major kinase involved in human NF-H KSP repeat region phosphorylation (*51*). In addition, we have also recently found that the two other motifs, KSPAEAK and KSPAEVK, can be phosphorylated by the MAPK family of kinases (unpublished data). In addition to phosphorylation found in the KS/TP motifs, a novel T phosphorylation site was found to be phosphorylated in vivo in a KVPTPEK motif, which occurs in the C-terminal tail domain. Again, on the basis of kinase consensus sequences, KVPTPEK would be expected to be phosphorylated by proline-directed kinases.

The LC/MS/MS methodology employed here has resulted in a remarkably high degree of characterization of the phosphorylation sites of human NF-H, previously described as the most hyperphosphorylated protein known (*1*). This can be contrasted to the much more complex and tedious protein chemistry procedures previously employed by our laboratory in the characterization of rat NF-H phosphorylation sites. It is anticipated that the LC/MS/MS methodology described here will be employed for the characterization of the phosphorylation sites of other in vivo and in vitro phosphorylated proteins.

ACKNOWLEDGMENT

We gratefully acknowledge Drs. Wayne Albers, Henry Fales, Harold Gainer, and Philip Grant for their helpful discussions and critical reading of the manuscript. We also acknowledge the assistance of Dr. Jeffrey Stebbins for his assistance in the running of the SEQUEST program.

[3] Modified Trypsin (1990) *Technical Bulletin 512*, Promega,Madison, WI 53711-5399.

REFERENCES

1. Pant, H. C., and Veeranna (1995) *Biochem. Cell Biol. 73*, 575−592.
2. Julien, J. P., and Mushinski, W. E. (1983) *J. Biol. Chem. 258*, 4019−4025.
3. Lees, J. F., Shneidman, P. S., Skuntz, S. F., Carden, M. J., and Lazzarini, R., (1988) *EMBO J. 7*, 1947−1955.
4. Elhanany, E., Jaffe, H., Link, W. T., Sheeley, D. M., Gainer, H., and Pant, H. C. (1994) *J. Neurochem. 63*, 2324−2335.
5. Yamasaki, H., Itakura, C., and Mizutani, M. (1991) *Acta Neuropathol. 82*, 427−434.
6. Mata, M., Kupina, M., and Fink, D. J. (1992) *Neurocytology 21*, 199−210.
7. O′Hara, O., Gahara, Y., Miyake, T., Teraoka, H., and Kitamura, T. (1993) *J. Cell Biol. 121*, 387−395.
8. DeWaegh, S. M., Lee, V. M. Y., and Brady, S. T. (1992) *Cell 68*, 451−463.
9. Eyer, J., and Peterson, A. (1994) *Neuron 12*, 389−405.
10. Goldstein, M. E., Sternberger, N. H., and Sternberger, L. A. (1987) *J. Neuroimmunol. 14*, 149−160.
11. Pant, H. C. (1988) *Biochem J. 256*, 665−668.
12. Krinks, M. H., Klee, C. B., Pant, H. C., and Gainer, H. (1988) *J. Neurosci. 8*, 2172−2182.
13. Lewis, S. E., and Nixon, R. A. (1988) *J. Cell Biol. 107*, 2689−2701.
14. Cook, L. C., Steinberger, N. H., Steinberger, L. A., Casonva, M. F., and Struble, R. G. (1986) *J. Neuropathol. Exp. Neurol. 45*, 56−64.
15. Zhang, H., Sternberger, N. H., Rubinstein, L. J., Herman, M. H., Binder, L. I., and Sternberger, L. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8045−8049.
16. Forno, L. S., Steinberger, L. A., Steinberger, N. H., Strefling, A. M., Swanson, K., and Eng, L. F. (1986) *Neurosci. Lett. 64*, 253−258.
17. Pollanen, M. S., Bergeron, C., and Weyer, L. (1994) *Acta Neuropathol. 88*, 1−6.
18. Manetto, N. H., Steinberger, N. H., Perry, G., Steinberger, L. A., and Gambetti, P. (1988) *J. Neuropathol. Exp. Neurol. 47*, 642−653.
19. Munoz, D. G., Grein, C., Perl, D. P., and Selkoe, D. J. (1988) *J. Neuropathol. Exp. Neuropathol. 47*, 9−18.
20. Sobue, G., Hashizumi, Y., Yasude, T., Mukai, E., Kumagai, T., and Trojanowski, J. Q. (1990) *Acta Neuropathol. 79*, 402−408.
21. Lee, V. M. Y., Carden, M., Schlaepfer, W., and Trojanski, J. (1987) *J. Neurosci. 7*, 3474−3488.
22. Xu, Z. S., Liu, W.-S., and Willard, M., (1990) *J. Neurosci. 10*, 1838−1846.
23. Xu, Z. S., Liu, W.-S., and Willard, M., (1992) *J. Biol. Chem. 267*, 4467−4471.
24. Betts, J. C, Blackstock, W. P., Ward, M. A., and Anderton, B. H. (1997) *J. Biol. Chem. 272*, 12922−12927.
25. Bennett, G. S., and Quintana, R. (1997) *J. Neurochem. 68*, 534−543.
26. Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fostis, T., and Mann, M. (1996) *Nature 379*, 466−469.
27. Mann, M., and Wilm, M. (1994) *Anal. Chem. 66*, 4390−4399.
28. Mortz, E., O'Conner, P. B., Roepstorff, P., Kelleher, N. L., Wood, T. D., McLafferty, F. W., and Mann, M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 8264−8267.
29. Eng, J. K, McCormack, A. L., and Yates, III, J. R. (1994) *J. Am. Soc. Mass. Spectrom. 5*, 976−989.
30. Yates, J. R. (1996) in *Methods in Enzymology, Vol. 271, High-Resolution Separation and Analysis of Biological Macromolecules, Part B, Applications* (Karger, B. L., and Hancock, W. S., Eds.) pp 351−377, Academic Press, San Diego, CA.
31. Yates, J. R., III, Eng, J. K., McCormack, A. L., and Schieltz, D. (1995) *Anal. Chem. 67* 1426−1436.
32. Shetty, K. T., Link, W. T., and Pant, H. C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6844−6848.
33. Veeranna, Shetty, K. T., Jaffe, H., Wang, J. H., and Pant, H. C. (1995) *J. Neurochem. 64*, 2681−2690.
34. Docemeci A., Floyd, C., and Pant H. C. (1990) *Cell Mol. Neurobiol. 10*, 369−382.
35. Stone, K. L., and Williams, K. R. (1993) in *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P., Ed.) pp 43−69, Academic Press, San Diego, CA.
36. Stoney, K., and Nugent, K., (1995) in *Techniques in Protein Chemistry VI* (Crabb, J. W., Ed.) pp 277−284, Academic Press, San Diego, CA.
37. Baldwin, S., Nugent, K., and Stoney, K. (1997) Presented at The Second Annual International Symposium of the Association of Biomolecular Resource Facilities (ABRF 97), Baltimore, MD, February 9−12.
38. Gillece-Castro, B. L., Arnott, D. P., Bier, M. E., Land, A. P., and Stults, J. T. (1995) Presented at the 43rd ASMS Conference on Mass Spectrometry and Allied Topics.
39. Chaudhary, T., and Land, A. (1997) *LCQ Application Report 260*, Finnigan MAT, San Jose, CA.
40. Land, A., Sanders, M., and Schwartz, J. (1996) *LCQ Application Report 255*, Finnigan MAT, San Jose, CA.
41. Wheeler, K., Mylchreest, I., Shoftstahl, J., Yates, J. R., III, and Eng, J. (1995) *TSQ Application Report 247*, Finnigan MAT, San Jose, CA.
42. Nuwaysir, L. M., and Stults, J. T. (1993) *J. Am. Soc. Mass. Spectrom. 4*, 662−669.
43. Jonscher, K. R., and Yates, III, J. R. (1997) *J. Biol. Chem. 272*, 1735−1741.
44. Lane, W. S., Land, A. P., and Jardine, I. (1995) *LCQ Application Report 248*, Finnigan MAT, San Jose, CA.
45. Nixon, R. A. (1993) *Brain Pathol. 3*, 29−38.
46. Hisanaga, S., Matsuoka, Y., Nishizawa, K., Saito, T., Inakagi, M., and Hirokawa, N. (1994) *Mol. Biol. Cell 5*, 161−172.
47. Lew, J., Baudette, K., Litwin, C. M. E., and Wang, J. H. (1992) *J. Biol. Chem. 267*, 13383−13390.
48. Shetty, K. T., Link, W. T., and Pant, H. C. (1993) *Proc. Natl. Acad Sci. U.S.A. 90*, 6844−6848.
49. Roder, H. M., and Ingram, V. M. (1991) *J Neurosci. 11*, 3325−3343.
50. Guan, R. J., Khatra, B. S., and Cohlberg, J. A. (1991) *J. Biol. Chem. 266*, 8262−8267.
51. Pant, A. C., Veeranna, Pant, H. C., and Amin, N. (1997) *Brain Res. 765*, 259−266.
52. Biemann, K., and Martin, S. A. (1987) *Mass Spectrom. Rev. 6*, 1.
53. Roepstorff, P., and Fohlman, J. (1984) *J. Biomed. Mass Spectrom. 11*, 601.
54. TSQ/SSQ 7000 Series Systems (1995) *Biochemistry Applications Software Manual, Revision B*, pp 5-3−5-8. Finnigan MAT, San Jose, CA.

BI972518U