Cytoplasmic O-GlcNAc Modification of the Head Domain and the KSP Repeat Motif of the Neurofilament Protein Neurofilament-H*

(Received for publication, April 19, 1996, and in revised form, May 31, 1996)

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Neurofilaments, the major intermediate filaments in large myelinated neurons, are essential for specifying proper axonal caliber. Mammalian neurofilaments are obligate heteropolymers assembled from three polypeptides, neurofilament (NF)-H, NF-M, and NF-L, each of which undergoes phosphorylation at multiple sites. NF-M and NF-L are known to be modified by O-linked N-acetylglucosamine (O-GlcNAc) (Dong, D. L.-Y., Xu, Z.-S., Chevrier, M. R., Cotter, R. J., Cleveland, D. W., and Hart, G. W. (1993) J. Biol. Chem. 268, 16679–16687). Here we further report that NF-H is extensively modified by O-GlcNAc at Thr53, Ser54, and Ser56 in the head domain and, somewhat surprisingly, at multiple sites within the Lys-Ser-Pro repeat motif in the tail domain, a region in assembled neurofilaments known to be nearly stoichiometrically phosphorylated on each of the ~50 KSP repeats. Beyond the earlier identified sites on NF-M and NF-L, O-GlcNAc sites on Thr19 and Ser36 of NF-M and Ser33 and Ser46 of NF-L are also determined here, all of which are localized in head domain sequences critical for filament assembly. The proximity of O-GlcNAc and phosphorylation sites in both head and tail domains of each subunit indicates that these modifications may influence one another and play a role in filament assembly and network formation.

Neurofilaments (NFs),† major 10 nm neuronal intermediate filaments, are the most abundant cytoskeletal components in large myelinated axons (1) and have been proven to be intrinsic determinants of axonal caliber (2–7), which in turn determines the axonal conduction velocity of action potentials (8). Abnormalities in NF organization may play a pivotal role in the etiologies of motor neuron diseases, such as amyotrophic lateral sclerosis (7, 9). Recent transgenic mouse models that over-express wild type subunits (10, 11) or express mutant forms (12) of NF subunits have closely mimicked the pathology of amyotrophic lateral sclerosis.

NFs are obligate heteropolymers assembled from three NF subunits, NF-H (115 kDa), NF-M (100 kDa), and NF-L (62 kDa) (13, 14). Structurally, each subunit has a globular head and a variable length tail domain separated by a 310-amino acid helical rod domain (15). The three subunits are synthesized in neuronal cell bodies, transported into axons, and extensively post-translationally modified in their head and tail domains. The aberrant mobility of NF-H and NF-M on SDS-polyacrylamide gel electrophoresis (PAGE) is caused by the high degree of phosphorylation of tail domains (16–19). For example, the rat NF-H tail contains 52 Lys-Ser-Pro (KSP) repeats (20), and the serine residue of each repeat can be phosphorylated yielding nearly stoichiometric modification post assembly in the internodal segments of axons (16–19). It has been proposed that as a consequence of repulsive force mediated by the negatively charged phosphates, phosphorylation of both NF-H and NF-M may contribute to the flexibility of the carboxyl tails and modulate NF density and interfilament spacing by altering cross-bridges between NFs or the interactions of NFs and other cytoskeletal components (1, 17, 21–25), whereas hypophosphorylation is correlated with the close packing of filaments in cell bodies, in dendrites, at nodes of Ranvier (23–25), and in vitro reassembled NFs (26).

The head domains of NF proteins are rich in serine and threonine residues and are also phosphorylated. Several sites have been identified on both NF-M and NF-L (27–29). The head domain itself is known to be crucial for the formation of 10 nm filaments (30–32). Besides phosphorylation, we have previously reported that NF-M and NF-L are also modified post-translationally with O-linked N-acetylgalactosamine (O-GlcNac) on serine and threonine residues (33). This type of glycosylation is the simplest protein modification with sugars (34). Initially discovered from a glycosylation study of murine lymphocytes (35), this modification has subsequently been found within the nucleoplasmic and cytoplasmic compartments of virtually all eukaryotic cells (36). O-GlcNac transferase, an enzyme responsible for the addition of GlcNac to the serine and/or threonine residues of peptides/proteins, and a cytoplasmic N-acetyl-β-D-glucosaminidase that selectively cleaves O-GlcNac from glycopeptides and proteins have both been purified (37, 38). The O-GlcNac modification (termed as O-GlcNAcylation) is thus both highly abundant and dynamic in a manner quite similar to phosphorylation (39–43), suggesting a regulatory role for O-GlcNAcylation in a variety of biological processes. In order to explore the possible role(s) of O-GlcNAcylation in NF functions, we extend further our original study.
and show that not only NF-M and NF-L but also NF-H are modified by O-GlcNAc. Most strikingly, we document that multiple serines in the ~50 KSP repeats of the tail domain of NF-H are O-GlcNAcylated, suggesting that it regulates the properties of the tail domain by directly competing and modulating phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Spinal cords were taken from 6-month-old or older male rats. UDP-[3H]galactose (38 Ci/mmol) was obtained from Amersham Corp. Bovine milk galactosyltransferase (GT'ase), chymotrypsin, and aprotinin were purchased from Sigma, and GT'ase (37 unit/ml) was pregalactosylated as described (36). Sequencing grade trypsin was from Boehringer Mannheim. Mixed monosaccharide standards were from Dionex. All other chemicals were of the highest quality commercially available.

Purification of NF Triplet Proteins—Total NFs comprising mainly NF triplet proteins were prepared from about 15 g of frozen rat spinal cord as described (33, 44, 45). The Triton X-100 insoluble pellet from repeated sucrose cushions was suspended in 20 mM sodium phosphate, pH 7.0, 8.0 mM urea, and 0.1% β-mercaptoethanol, filtered through a 0.45-μm filter, and loaded onto a Fractogel HW-40C column (1.5 × 40 cm, Tosoh Corp). The column was washed with 20 mM sodium phosphate and 0.1 M sodium perchloride, pH 6.9, and eluted with a linear CH3CN gradient: 0–5 min, 0–20%; 5–60 min, 20–60%; 60–80 min, 60–100%. NF-H was separated again on the second dimension RP-HPLC and each radioactive peptide was collected on the first dimension RP-HPLC, was further purified by a third dimension RP-HPLC on a CarboPAC-MA1 column using 1 mM of 2-deoxyglucose as an internal standard.

Preparation of Chymotryptic Fragments of NF-H Head Domain—Purified NF-H was first digested with chymotrypsin (weight ratio, 200:1) in 0.1% ammonium bicarbonate, pH 8.25, 10% CH3CN, for 2 h at room temperature. The sample was centrifuged in a Microcon-30 (Amicon), and the flow through containing small chymotryptic fragments was lyophilized.

Trypsin Digestion of NF-H—Purified NF-H (0.48 mg) was first digested with trypsin (weight ratio, 20:1) in 100 mM Tris, pH 8.5, and 10% CH3CN at 37 °C for 18 h, and the digestion was continued for another 10 h with an addition of the same amount of trypsin. The reaction was stopped by adding 2 μl of aprotinin (2 mg/ml). The trypsinated peptides were desalted through two Sep-Pak C18 cartridges (Waters), eluted with 60% CH3CN, and dried.

GT'ase Labeling of NF-H Peptides and isolation of O-GlcNAcylated Glycopeptides—Peptides from chymotrypsin digestion of NF-H were labeled with UDP-[3H]galactose and GT'ase and subjected to mild alkali-induced elimination (35, 46). The labeled mixture was precipitated with 8 volumes of cold acetone. The pellet was resuspended in gel sample buffer and analyzed by SDS-PAGE.

Sugar Analysis—Purified NF subunits were labeled with UDP-[3H]galactose and GT'ase and subjected to mild alkali-induced elimination (35, 46). The labeled mix was precipitated with 8 volumes of cold acetone. The pellet was resuspended in gel sample buffer and analyzed by SDS-PAGE.

Gas-Phase Sequencing and Manual Edman Degradation Sequencing of [3H]Galactose-labeled Glycopeptides—Following the first, second, or third dimension RP-HPLC, purified glycopeptides of NF proteins were sequenced by automated Edman degradation in a Model 470A gas-phase sequencer (Applied Biosystems, Inc.). M. E. sequencing of glycopeptides was performed by covalent coupling of peptides to Sequelon-AA membranes (Milligen/Biosearch of Millipore), followed by repeated coupling of phenyl isothiocyanate and trifluoroacetic acid extraction as described (47), except that the membrane (or filter) from each cycle was extracted and washed with 0.5 ml of trifluoroacetic acid and then washed with methanol twice, and all extraction and washes were performed, dried, and neutralized with 300 μl of 1.0 M tris, pH 8.8, before scintillation counting.

SDS-PAGE and Autofluorography—NF proteins were analyzed by 8.0% SDS-PAGE (48). Gels were either stained with Coomassie Brilliant Blue or treated with EnHANCE (DuPont NEN), dried, and fluorographed.

Immunoblot Assay—Protein concentration was determined by Amido Schwarz dye binding procedure (49) using bovine serum albumin as a standard.

RESULTS

NF Triplet Proteins Are [3H]Galactose Labeled by GT'ase—A common and sensitive method to detect O-GlcNAcylation of proteins is to covalently label the protein-bound sugar with UDP-[3H]galactose and GT'ase (36, 46). Total NFs, comprised mainly of the three NF subunits, NF-H (200 kDa), NF-M (145 kDa), and NF-L (62 kDa), were isolated from rat spinal cord (Fig. 1A, lanes 1, 2, and 3). Total NFs and each purified subunit were separately labeled with UDP-[3H]galactose and GT'ase, analyzed by SDS-PAGE, and fluorographed. As shown in Fig. 1B, NF-M and NF-L were strongly radiolabeled by [3H]galactose both in the total cytoskeletal pellet (Fig. 1B, lane 4) and in the purified form (Fig. 1B, lanes 1 and 2). Purified NF-H and the tail domain of NF-H could only be labeled with GT'ase if they were first denatured by treating with 0.5% SDS and boiling prior to an extended incubation with GT'ase. Even so, NF-H was only weakly la-
beled in the purified form (Fig. 1B, lane 3), and the lengthy incubation resulted in several labeled bands around 50 kDa that are degradation products of NF-H (see below). The carboxyl-terminal tail domain of NF-H, consisting of 52 KSP repeats, was labeled even more weakly (data not shown). The relatively weak \(^{3}H\)galactose incorporation into the purified NF-H is due, at least in part, to the tendency of purified NF-H to self-aggregate during GT'ase labeling. The labeling of NF-H was improved in the total NF s fraction (Fig. 1B, lane 4). Relative to NF-M and NF-L, purified NF-H can be labeled more efficiently at 4°C or on ice than at room temperature or at 37°C because the aggregation of NF-H in the GT'ase labeling reaction is very severe at 37°C. GT'ase labeling of NF-H was also somewhat enhanced by dephosphorylation prior to labeling (data not shown), also suggesting that the phosphates may block accessibility of GT'ase. That the addition of \(^{3}H\)galactose to each NF subunit represented O-GlcNAcylation was demonstrated by the resistance to peptideN-glycosidase F treatment and virtually 100% accessibility to mild alkali-induced \(\beta\)-elimination (see below).

**NF-H Is Modified with Unelongated Monosaccharides, O-GlcNAc**—In order to identify the sugar residues modified on NF-H, purified NF-H was labeled with GT'ase, subjected to alkaline-induced \(\beta\)-elimination, and analyzed on a Sephadex G-50 gel filtration column (Fig. 2). The \(\beta\)-elimination products chromatographed as a single Vi peak on the column (Fig. 2A). The Vi peak (fractions 28–31) was run on a high resolution gel filtration TSK Fractogel column. The labeled sugar moieties migrated as a single peak between standards of one and two filtration TSK Fractogel column. The labeled sugar moieties and NF-H; lane 3, NF-L; lane 2, NF-M; lane 3, NF-H; lane 4, total NFs. The positions of NF-H (200 kDa), NF-M (145 kDa), and NF-L (62 kDa) are indicated in the middle.

**Fig. 1. NF triplet proteins are \(^{3}H\)galactose-labeled with GT'ase.** Each purified NF subunit and total NF proteins were separately labeled with GT'ase and resolved on a 8.0% SDS-PAGE. A, Coomassie Brilliant Blue staining. B, fluorography of \(^{3}H\)galactose-labeled NF proteins. Lane 5, protein standards; lane 1, NF-L; lane 2, NF-M; lane 3, NF-H; lane 4, total NFs. The positions of NF-H (200 kDa), NF-M (145 kDa), and NF-L (62 kDa) are indicated in the middle.

**Fig. 2. NF-H is modified by a single monosaccharide GlcNAc.** Purified NF-H was labeled with GT'ase, and the \(\beta\)-elimination products were further characterized as described under “Experimental Procedures.” A, G-50 profile of \(^{3}H\)galactose-labeled \(\beta\)-elimination products. \(V_{i}\), void volume; \(V_{o}\), included volume. B, TSK Fractogel chromatography. Arrows indicate the elution positions of \(^{3}H\)galactose-labeled GlcNAc polymers. The numbers of GlcNAc residues are also shown. C, HPAEC-PAD analysis of \(\beta\)-elimination products on a Dionex CarboPAc-MA1 column. Arrows 1 and 2 denote elution positions of authentic Galβ1-3GalNAcitol and Galβ1-4GalNAcitol, respectively.

and 0.1 mol of O-GlcNAc previously found for NF-M and NF-L, respectively (33). This establishes a minimum estimate because O-GlcNAc may be removed during NF purification (50 mM GlcNAc was always included in the homogenization buffer, but this only partially inhibits both cytosolic N-acetyl-\(\beta\)-D-glucosaminidase (38) and lysosomal hexosaminidases (data not shown)).

The Head Domain of NF-H Has a Single O-GlcNAc-modified Peptide—Identified O-GlcNAc sites on both NF-M and NF-L are predominantly localized on the head domains (33). Mild digestion of NF-H with chymotrypsin results in the fragmentation of head and rod domains of NF-H and leaves the carboxyl terminus (beginning at Ile\(^{632}\)) (19, 50) intact. Purified NF-H was thus digested with chymotrypsin, and peptides from the head and tail domains were isolated and labeled with UDP-\(^{3}H\)galactose and GT'ase as described under “Experimental Procedures.” Radiolabeled peptides were separated by RP-HPLC on a C18 column. As shown in Fig. 3, only a single chymotryptic peptide from the head and rod domains of NF-H was labeled (bottom panel), and this peak corresponded to the major mass peak at elution time of 37 min (top panel). Gas-phase sequencing revealed the peptide sequence of 51ARTS-VSSVSASPSRF\(^{55}\), located in the middle of the head domain of NF-H (20). MED sequencing, a method successfully used to determine O-GlcNAc sites on glycopeptides of both NF-M and
NF-L (33), repeatedly failed to identify the site(s) of O-GlcNAcylation on this peptide, primarily because the peptide did not efficiently attach covalently to the membrane. The reason(s) for this remains unclear.

Identification of Thr53, Ser54, and Ser56 of the Head Domain and Multiple Sites in the KSP Repeats of the Tail Domain as Major O-GlcNAc Attachment Sites of NF-H—In order to examine the natural sites of O-GlcNAcylation of NF-H protein, purified NF-H was first digested with trypsin and then labeled with UDP-[3H]galactose by GT'ase as performed previously on both NF-M and NF-L (33). Radiolabeled peptides were separated by RP-HPLC on a C18 column as described under "Experimental Procedures." As seen in Fig. 4, a minimum of eight [3H]galactose-labeled glycopeptide peaks were resolved. Radioactivity essentially covered the entire elution profile from 17 to 120 min. In a separate experiment, when trypsinized and GT'ase-labeled NF-H peptides were redigested with 10:1 ratio of protein to trypsin and chromatographed on the C18 column, a similar number of peaks were still observed, a finding suggesting that the multiple peaks do not represent incomplete digestion products. Sequential trypsin digestion followed by UDP-[3H]galactose and GT'ase labeling of dephosphorylated NF-H also generated about a dozen tritium peaks (data not shown), further confirming the multiple O-GlcNAcylation of NF-H. By comparing the total [3H]galactose incorporation of the head domain (peptide ARTSVSSVASPSRT in Fig. 3) with that of the tryptic carboxyl domain and the amount of radioactivity in head domain (peak 4, Fig. 4, see below) with the rest of peaks (i.e., that in KSP repeats), we estimate that the ratio of O-GlcNAc on the tail domain versus the head domain is more than 10 to 1.

Peaks 1–8 were individually further chromatographed on a second dimension C18 column with a different solvent system as described. Each then generated one or more radioactive peaks (Fig. 5). Several major radioactive glycopeptide peaks from the second dimension RP-HPLC (Fig. 5) were subjected to both gas-phase sequencing and MED sequencing, and the data are shown in Fig. 6. All but Peak 4 yielded a radioactive peak eluted at cycle number 1 from MED sequencing, indicating that the first amino acid residue of these tryptic glycopeptides is either serine or threonine. Because trypsin cleaves proteins at the carboxyl side of lysine and arginine, each glycopeptide has a (K/R)(S/T) motif. Gas-phase sequencing of Peaks 3a and 3b contained a mixture of peptides, with major sequence of 114QLEAHNT120 and 201FAQEA205 both from the rod domain of NF-H (20), respectively, but also revealed minor sequences of SPA(T/S)VK and SPVTVK, which are parts of the KSP repeats. MED sequencing showed the released radioactive peak is at cycle number 1 for Peak 3b and 3c (Fig. 6) and failed to reveal the peak number for Peak 3a, suggesting that the first serine on SPA(T/S)VK and SPVTVK peptides are the ones modified by O-GlcNAc. No gas-phase sequence could be obtained for the Peaks 2a, 2b, 5, 6a, and 7a, but they all showed radioactive release at cycle number 1, indicating that the tritium is released from serine residues of other KSP repeats. Therefore, some or all serines on the KSP repeats of the tail domain of NF-H are modified with O-GlcNAc.

The peptide sequence from gas-phase sequencing of Peak 4a
is $^{53}$TSVSSVSA(SPESR)$_{64}$, and MED sequencing showed tritium released at cycle number 2, demonstrating Ser 54 is modified by O-GlcNAc-$[^{3}H]$galactose (Fig. 6). MED sequencing of Peak 4b also revealed radioactivity released at cycle number 4, and two minor peaks at cycles 1 and 2 (Fig. 6), corresponding to Ser$_{56}$, Thr$_{53}$, and Ser$_{54}$ of the peptide $^{53}$TSVSSVSA(SPESR)$_{64}$. Peak 4c released a strong single tritium peak at cycle number 4 (Fig. 6), suggesting the modification of the peptide. No amino acid sequence could be obtained for both peptides of Peak 4b and 4c; however, based on the very close elution profile on the first dimension RP-HPLC, a strong and single $[^{3}H]$galactose labeling peak of chymotryptic peptide 51ARTSVSSVSA(SPESR)$_{64}$ and good match between MED sequencing data and the peptide sequence, it thus suggests that Thr$_{53}$, Ser$_{54}$, and Ser$_{56}$ of NF-H are all modified with O-GlcNAc. Peak 4a represents $^{53}$TS(O-GlcNAc)VSVSSVSA(SPESR)$_{64}$, which eluted at the earliest time, Peak 4b either contains all three O-GlcNAc sites, 53T(S(O-GlcNAc))VS(S(O-GlcNAc))VSSVSASPSR$_{64}$, and Peak 4c has only one O-GlcNAc site at Ser$_{56}$ of $^{53}$TSVS(O-GlcNAc)VSASPSR$_{64}$. Therefore, Peak 4a, 4b, and 4c likely represent the different forms of O-GlcNAcyla-tion (or a combination of different O-GlcNAcylation and phosphorylation) of a common chymotryptic peptide $^{53}$TSVSSVSA(SPESR)$_{64}$. Thr$_{19}$ and Ser$_{34}$ of NF-M Are Probable Sites of O-GlcNAc Modification—We have previously reported that NF-M and NF-L are extensively modified by O-GlcNAc. Each revealed four major $[^{3}H]$galactose-labeled peptides, and two O-GlcNAc sites from both NF-M and NF-L (Thr$_{48}$ and Thr$_{431}$ of NF-M and Thr$_{21}$ and Ser$_{27}$ of NF-L) were identified (33). Peak A was further purified by a second and third dimension RP-HPLC according to “Experimental Procedures” and then subjected for gas-phase sequencing. The amino acid sequence of the purified peptide is $^{31}$SGYSTAR$_{37}$ (52) (Fig. 8). The site of O-GlcNAc attachment was determined to be Ser$_{34}$ of NF-L by measurement of tritium counts released from each

![Fig. 5. Second dimension RP-HPLC analysis of tryptic and GT'ase labeled NF-H peptides. Peaks 1–8 from the first dimension RP-HPLC in Fig. 4 were further purified on the second dimension RP-HPLC according to the “Experimental Procedures.” Tritium profiles from each 1-min fraction are shown. Each tritium peak is numbered followed by letter a, b, or c in the order of peak elution.](image)

![Fig. 6. MED sequencing of second dimension peptide peaks of NF-H. MED sequencing of Peaks 2a, 2b, 3b, 4a, 4b, 4c, 5, 6a, and 7a was performed as described under “Experimental Procedures.” Tritium counts released from each cycle and peptide sequence of Peaks 4a, 4b, and 4c are shown.](image)
O-GlcNAcylation of NF-H

**DISCUSSION**

Combined with our earlier effort (33), we have now shown that NF triplet proteins, NF-H, NF-M, and NF-L, are modified by O-GlcNAc in vivo to stoichiometries of at least 0.3, 0.15, and 0.1 mol GlcNAcmol of protein, respectively. We have demonstrated that NF-H is modified by O-GlcNAc at three sites of the head domain, Thr53, Ser54, and Ser55, and at multiple serine residues in the KSP repeats of the carboxyl tail domain, although we would caution that the exact number of KSP repeats modified by O-GlcNAc is hard to determine (Fig. 10). In addition, four sites both in NF-M and NF-L have been identified (Fig. 10). As shown in Fig. 10B, a stretch of less than 40 amino acids of the head domain of NF-M has at least three O-GlcNAc sites and eight phosphorylation sites identified by in vitro labeling with protein kinase A or C (29), or by in vivo labeling (27). Intriguingly, the known O-GlcNAc and phosphorylation sites in the head domain are in close proximity but not overlapping. Given the proximity of the O-GlcNAc sites to the phosphorylation sites in the head domain and importance of the head domain in filament assembly (30–32), it is plausible that modification by O-GlcNAc may also play a role in regulating the timing or extent of filament assembly. This regulation could be exerted by either direct or indirect (e.g., by affecting phosphorylation) influence on the structure of the head domain, which in turn modulates assembly of NFs.

The co-existence of both phosphate and O-GlcNAc on the same NF subunits adds to the growing list of proteins that exhibit such a pattern. Nearly one hundred intracellular phosphoproteins are so far also known to bear O-GlcNAc, and more than thirty in vivo O-GlcNAc attachment sites have been mapped (33, 39, 53). Among all mapped O-GlcNAc sites, including all sites identified here from NF triplet proteins, most have a proline nearby, most commonly on the amino-terminal side of the modified serine or threonine residue. This has revealed a consensus of PXO(S/T) motif (X is usually a hydrophobic residue) similar to those used by proline-directed and growth factor kinases (39). In addition, in many cases, like the identified sites on NFs, there is also a valine or glutamine residue close to the O-GlcNAc site. Therefore, although a precise consensus sequence cannot be drawn from all available sites, there is a common motif for O-GlcNAcylation: PVQXO(S/T).

Although the stoichiometry of O-GlcNAcylation on isolated NFs is low compared with phosphorylation, it seems likely that only a subset of NF subunits is modified by O-GlcNAc, and the stoichiometry in those subunits is probably much higher. Almost all (>99%) of the assembled NFs examined here represent NFs in myelinated internodal regions known to be nearly stoichiometrically phosphorylated in the KSP repeat domain (25). In contrast, the KSP repeats of subunits in cell bodies, dendrites, and nodes of Ranvier are unphosphorylated, but account for ~1% of NFs in the neuron as judged by NF density and the relative volumes of the respective compartments (23–25). Thus, the NF proteins isolated here from rat spinal cord are almost certainly a mixed population of highly phosphorylated, partially phosphorylated, partially O-GlcNAcylated, or fully O-GlcNAcylated proteins. Thus, it seems plausible that for those subunits modified by O-GlcNAc, the true stoichiometry may be much higher (estimated by the measured stoichiometry divided by the fraction of protein from the unmyelinated domains). Further, the exact level of O-GlcNAcylation may be even higher in view of the dynamic nature of the modification and the presence of strong activities of both cytosolic N-acetyl-β-D-glucosaminidase (38) and lysosomal hexosaminidases (33, 38, 54) that may act during the purification process.

domain of rat NF-L are O-GlcNAcylated based on gas-phase sequencing, MED sequencing, and mass spectrometry.

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**Fig. 7. MED sequencing of Peak 1 and 2 from NF-M.** Peak 1 and 2 from trypsin digestion of NF-M (33) were further purified by RP-HPLC, and MED sequencing was performed to determine the site of O-GlcNAcylation. A and B, tritium counts released from each cycle of MED sequencing and peptide sequences derived from tryptic head domain of NF-M (51).

**Fig. 8. Identification of Ser54 as an O-GlcNAc attachment site in NF-L.** Peak A, the first radioactive peak on the first RP-HPLC dimension of NF-L (33), was further purified by two more dimensions of RP-HPLC with different solvent conditions and sequenced. The amino acid sequence of the purified peptide is shown, and the site of O-GlcNAc attachment site is determined by the counts released from each cycle of MED sequencing.

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depth of NF-L (33) were further purified by RP-HPLC, and MED sequencing was performed to determine the site of O-GlcNAcylation. A and B, tritium counts released from each cycle of MED sequencing (Fig. 8). The amino acid sequence of the purified peptide is shown, and the site of O-GlcNAc attachment site is determined by the counts released from each cycle of MED sequencing. (Fig. 9)
NFs are dynamic structures (55, 56), the dynamic process involves the continuous turnover of NFs and lateral subunit exchange and involving incorporation of new subunits (55). Because NFs are also known to be highly phosphorylated post-assembly in axons and less phosphorylated in cell bodies and proximal axons (23, 25), it is possible that O-GlcNAc attachment was determined by MED sequencing. A and B, tritium profile on second dimension RP-HPLC of original and chymotryptic digested peptide Peak D, respectively. C and D, tritium counts released from each cycle of MED sequencing for original and digested Peak D, respectively.

Fig. 9. Identification of Ser48 as a major O-GlcNAc attachment site in NF-L. Peak D, the fourth and most prominent radioactive peak on the first RP-HPLC dimension of NF-L (33), was digested by chymotrypsin, resolved on the second dimension RP-HPLC, and the site of O-GlcNAc attachment was determined by MED sequencing. A and B, tritium profile on second dimension RP-HPLC of original and chymotryptic digested peptide Peak D, respectively. C and D, tritium counts released from each cycle of MED sequencing for original and digested Peak D, respectively.

Fig. 10. Schematic representation of O-GlcNAc modification and phosphorylation sites of NF triplet proteins. A, protein structure, O-GlcNAcylation, and phosphorylation sites of NF-H, NF-M, and NF-L. Helical rod domain (bars with dots), head domain (bars with wavy lines), and all previous (33) or currently identified sites of O-GlcNAcylation and phosphorylation (19, 27–29, 44, 45, 50) are shown. The approximate localization of amino acid numbers are based on the sequence data from rat NF-H (20), NF-M (51), and NF-L (52). B, identified in vivo O-GlcNAc sites and in vivo/in vitro phosphorylation sites (29) of rat and mouse NF-M, respectively. Peptide sequence from amino acid residues 17 to 53 of NF-M are identical between mouse and rat (51, 58).

NFs are dynamic structures (55, 56), the dynamic process involves the continuous turnover of NFs and lateral subunit exchange and involving incorporation of new subunits (55). Because NFs are also known to be highly phosphorylated post assembly in axons and less phosphorylated in cell bodies and proximal axons (23, 25), it is possible that O-GlcNAc is added to NF proteins by O-GlcNAc transferase (37) right after their synthesis and prior to segmental assembly into filaments and transport into the distal part of axons. Subsequently, a N-acetyl-β-D-glucosaminidase (38) removes the sugars, and phosphates are added by protein kinases (5, 56). Phosphorylation of the carboxyl domains of NF-H and NF-M has been proposed to modulate interfilament spacing (21–23) by interacting with adjacent filaments and other organelles, thereby establishing a wider filament spacing, perhaps through repulsion between the adjacent filaments as a result of the highly negatively charged phosphates (17, 22–24). By replacing phosphates with O-GlcNAc, the interactions between filaments may switch from a repulsive one to an associative one, leading to close packing of filaments, for example, in nodes of Ranvier.
Therefore, it seems likely that organization of NFs is regulated by kinase/phosphatase (5, 56) and O-GlcNAc transferase/N-acetyl-β-D-glucosaminidase (37, 38). The dynamic O-GlcNAcylation (39, 40) and phosphorylation (56) could therefore modulate proper assembly of NFs and maintain the dynamic nature of filaments, and abnormalities in either could contribute to any of the many motor neuron diseases in which NFs accumulate aberrantly (5, 57).

Acknowledgments—We thank Dr. Wu-Schyong Liu of Protein/Pep-tide Facility of Department of Biological Chemistry at the Johns Hopkins University School of Medicine for expertise on sequencing of peptide and amino acid composition analysis. We also thank Dr. M. Daniel Lane for use of the HPLC system.

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