In Vivo and in Vitro Phosphorylation at Ser-493 in the Glutamate (E)-segment of Neurofilament-H Subunit by Glycogen Synthase Kinase 3β*

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Neurofilament (NF), a major neuronal intermediate filament, is composed of three subunits, NF-L, NF-M, and NF-H. All three subunits contain a well conserved glutamate (E)-rich region called “E-segment” in the N terminus of the tail region. Although the E-segments of NF-L and NF-M are phosphorylated by casein kinases, it has not been observed in NF-H. Using mass spectrometric analysis, we identified phosphorylation of the E-segment of NF-H, prepared from rat spinal cords, at Ser-493 and Ser-501 in the Ser-Pro sequences. The E-segment kinase was isolated from rat brain extract using column chromatography and identified as glycogen synthase kinase (GSK) 3β. GSK3β was shown to phosphorylate at Ser-493 in vitro by phosphopeptide mapping and site-directed mutagenesis, and in vivo in HEK293 cells using the phospho-Ser-493 antibody, but did not phosphorylate Ser-501. GSK3β preferred Ser-493 to the KSP-repeated sequences for phosphorylation sites in the NF-H tail domain. Moreover, Ser-493 was a better phosphorylation site for GSK3β than other proline-directed protein kinases, Cdk5/p35 and ERK. GSK3β in the spinal cord extract was associated with NF cytoskeletons. Taken together, we concluded that Ser-493 in the E-segment of NF-H is phosphorylated by GSK3β in rat spinal cords.

Neurofilaments (NFs), major neuronal intermediate filaments, are the most abundant cytoskeletal element in the axon (1–3). NFs are required for the radial growth of axons, as shown by the reduced axonal caliber in mice and quail lacking NFs (4–6). NFs are heteropolymers of three subunits: NF-L, NF-M, and NF-H with molecular masses of 66, 95, and 115 kDa, respectively (7–9). Like other intermediate filament proteins, each NF subunit has an α-helical rod domain responsible for formation of 10-nm filaments. The rod domain is flanked by an N-terminal head domain, involved in the regulation of filament assembly and disassembly, and a C-terminal tail domain. The tail domain of larger molecular mass subunits, NF-M and NF-H, constitutes side arms extruding from the core filament (10) and are thought to be the region that interacts with other NFs or other cellular structures (11, 12).

NFs are one of the most phosphorylated proteins in neurons, and NF functions are suspected to be modulated by phosphorylation. The phosphorylation of the head domain by second messenger-dependent protein kinases induces filament disassembly in vitro (13–15). Nascent NF-L is phosphorylated at Ser-55 by PKA until incorporation into the axonal NF network (16) and at Ser-57 by calcium/calmodulin-dependent protein kinase II (CaMKII) at apical dendrites of the pyramidal neurons in a hippocampal slice culture when long term potentiation is induced (17). The long C-terminal tail domains of two larger subunits, NF-M and NF-H, are highly phosphorylated in axons. The phosphorylation occurs at the Lys-Ser-Pro (KSP) sequences, repeated ~10 times in NF-M and 50 times in NF-H. The KSP repeats were reported to be phosphorylated by the proline-directed protein kinases, Cdk5, ERK, stress-activated protein kinase, and GSK3 (18–23). This phosphorylation is thought to regulate the functions of the side arms on interactions between NFs or NF and other cellular structures (12, 24), the rate of NF transport (25), and proteolysis of NF proteins (26).

In addition to phosphorylation of the KSP repeat, the tail region of the NF subunit proteins is characterized by a glutamate (E)-rich region called the “E-segment” in the N terminus of the tail region. The importance of the E-segment in filament organization is suggested by the removal of the E-segment from NF-L by thrombin, which results in a ribbon-like structure, not an intermediate-size filament (27). There have been several reports describing phosphorylation of the E-segment, although the role of the phosphorylation remained unknown. Ser-473 in the E-segment of NF-L is phosphorylated in rat spinal cord and in vitro by casein kinase (CK) II (28, 29). The E-segment of the chicken NF-M is phosphorylated at five sites in vivo and by NF-associated CKI in vitro (30–32). If phosphorylation of the
E-segment plays an important role in filament formation, the E-segment of NF-H might also be phosphorylated as it has several consensus phosphorylation sequences for CKs. However, there has been no report on phosphorylation of the E-segment of NF-H.

We investigated the phosphorylation of the E-segment of NF-H and found that Ser-493 and Ser-501 in an SP motif in the NF-H E-segment were phosphorylated in vivo. We purified an E-segment kinase from rat brains and identified it as GSK3β. The preferred in vitro and HEK293 cell phosphorylation site for GSK3β was shown to be Ser-493, one of the in vivo phosphorylation sites, rather than the KSP repeats. A part of GSK3β was co-eluted with the NF fraction when spinal cord extract was subjected to gel filtration. These results indicate that Ser-493 in the E-segment of NF-H is phosphorylated by GSK3β in vivo.

**Materials and Methods**

**Chemicals, Antibodies, and Protein Kinases—** Ollomucine, an inhibitor of CdK5 and ERK2, was purchased from Calbiochem (San Diego, CA) and butyrolactone I, an inhibitor of CdK5, was provided by Dr. A. Okuyama (Banyu Pharmaceutical Research Institute in collaboration with Merck Research Laboratories, Tsukuba, Japan) (33). Anti-FLAG (M2) and anti-NF-H (SM33) monoclonal antibodies were purchased from Sigma and Sternberger Monoclonals Inc. (Baltimore, MD), respectively. Anti-rat GSK3β antiserum was described previously (34). ERK2 was purchased from New England Bioslabs. CdK5/p35 was prepared from SF9 cells transfected with mouse CdK5 and p35 cDNA. All other reagents were of analytical grade.

**Preparation of the Antibody against Phosphorylated Ser-493 of NF-H (pS493)—The two synthesized peptides, “CEAATTSPPAEE” and “CEAATTPSPPAEE” (where pS is phosphoserine), were chemically synthesized. The C-EEATTPSPPAEE peptide was conjugated to keyhole limpet hemocyanin through the cysteine residue attached to the N terminal and used for immunization in rabbits. The anti-pS493 serum was affinity purified on the columns that bound the nonphosphorylated C-EEAATTTPSPPAEE.

**Preparation of the Antibody against Phosphorylated Ser-493 of NF-H (pS493)—The two synthesized peptides, “CEAATTSPPAEE” and “CEAATTPSPPAEE” (where pS is phosphoserine), were chemically synthesized. The C-EEATTPSPPAEE peptide was conjugated to keyhole limpet hemocyanin through the cysteine residue attached to the N terminal and used for immunization in rabbits. The anti-pS493 serum was affinity purified on the columns that bound the nonphosphorylated peptide negatively and phosphorylated peptide positively.

**Preparation and Dephosphorylation of NFs—** Albino Wistar/ST rats (Japan-SLC, Tokyo, Japan) were anesthetized under diethyl ether vapor and decapitated. Spinal cords were homogenized in an equal volume (v/v) of a homogenization buffer (100 mM Pipes, pH 6.8, 5 mM EGTA, 1 mM MgCl2, 1 mM dithiothreitol (DTT), 0.4 mM Pefabloc SC, 10 µg/ml leupeptin) with a Polytron homogenizer. The homogenate was centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was fractionated using a Sepharose CL-4B gel filtration column (0.8 × 27 cm; Amersham Biosciences) with 20 mM Pipes, pH 6.8, 2 mM EDTA, 1 mM MgCl2, 0.1% Tween, 1 mM DTT, 0.02 mM Pefabloc SC (Merck, Darmstadt, Germany) and 1 µg/ml leupeptin. The void volume fractions were used as a NF fraction. NF was dephosphorylated with E. coli alkaline phosphatase (WAKO Chemicals, Osaka, Japan) for 2 h at 37 °C after dialysis against 50 mM Tris-HCl, pH 8.8, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM MgCl2, 0.15 µM NaCl, 1 mM DTT, 0.02 mM Pefabloc SC, 1 µg/ml leupeptin.

**Lysyl-endopeptidase Digestion of NF-H and Mass Spectrometric Analysis—** NF-H resolved by SDS-PAGE was excised from gels and washed with 25% isopropl alcohol and subsequently 10% methanol. After washing with acetone for 15 min at room temperature, the gels were dried and NF-H was digested by incubation with 0.1 unit of lysyl-endopeptidase (Wako Chemicals) in 500 µl of 10 mM (NH4)2CO3, pH 8.4, overnight at 30 °C. After lyophilization, peptide fragments dissolved in 50% CH3CN and 0.1% trifluoroacetic acid were mixed with an equal volume of 10 mg/ml α-cyan-4-hydroxycinnamic acid in 50% CH3CN and 0.1% trifluoroacetic acid as a matrix and analyzed for their mass by a Voyager Elite DE-STR (Perseptive Biosystems, Palo Alto, CA). Phosphorylated amino acids in the phosphorylated peptide were determined by the MS/MS procedure (quadrupole-time of flight, Micromass, Manchester, UK). The mass spectrometric data were assigned to amino acid sequences by using the MASCOT on-line service (www.matrixscience.com).

**Preparation of the NF-H Tail Domain and E-segment Expressed in E. coli—** The tail domain (425–1072 amino acids) expression vector was constructed from rat NF-H cDNA (9). The ApoI fragment of NF-H cDNA was cloned into the EcoRI site of pET23a (+) and, to adjust the reading frame, digested with BamHI and Nhel, blunt ended, and self-ligated. The E-segment (425–507 amino acids) expression vector was constructed from the tail domain expression vector by polymerase chain reaction (PCR). Mutations S493 to S493A, S501A to S493/501A were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The tail domain and E-segment were expressed in E. coli BL21 (DE3) pLysS (35) in the presence of 0.5 mM isopropl-β-D-thiogalactopyranoside. The tail domain was purified by a Hi-Trap heparin column exchange column (1.6 × 25 cm; Amersham Biosciences) and the E-segment was purified by a HiTrap Q anion exchange column (1.6 × 25 cm; Amersham Biosciences) from E. coli extract.

**Measurement of the Kinase Activity—** The kinase activity was measured in Buffer A (20 mM MOPS, pH 6.8, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM MgCl2) containing 0.1 mM ATP and 0.1 mM cAMP [γ-32P]ATP using 0.2 mg/ml tail domain or 0.1 mg/ml E-segment as substrate for 30 min at 35 °C. The reaction was stopped by addition of 4× LSB (Laemmli’s sample buffer) (35). After SDS-PAGE on a 7.5% polyacrylamide gel for the tail domain or 15% for the E-segment, phosphorylation was measured with a BAS 2000 bioimage analyzer (FujiFilm, Tokyo, Japan).

**Purification of the E-segment Kinase from Rat Brain Extracts—** Brains of 10-week-old rats were homogenized in 3 volumes (v/w) of Buffer A (0.32 M sucrose, 10 mM Pipes, pH 6.8) prior to centrifugation at 100,000 × g at 4 °C. The supernatant was loaded on a Superose 6 HR 10/30 gel filtration column (Amersham Biosciences) equilibrated with Buffer B. The E-segment kinase activity was eluted as a single peak at around 0.75 M NaCl with a linear gradient of 0–1.0 M NaCl in Buffer B. The fractions containing the E-segment kinase activity were collected and applied to a Superox 6 HR 10/30 gel filtration column (Amersham Biosciences) equilibrated with Buffer B. The E-segment kinase activity was detected as a peak at around 40–50 kDa.

**Immunoprecipitation—** An anti-GSK3β serum was added to the kinase fraction and incubated for 1 h at 4 °C. After addition of Protein A-Sepharose CL-4B (Amersham Biosciences), the mixture was further incubated for 1 h at 4 °C. After washing with Buffer A four times, the kinase activity bound to Sepharose beads was assayed as described above.

**Two-dimensional Phosphopeptide Map Analysis and Phosphoamino Acid Analysis—** The tail domain or E-segment phosphorylated by GSK3β was digested by lysyl-endopeptidase. The digested peptides were subjected to two-dimensional phosphopeptide map analysis using an HPTLC gel (Merck) according to the methods of Boyle et al. (36). Electrophoresis was performed at pH 1.9 and ascending chromatography was performed in isobutycylic acid buffer.

The 32P-labeled peptide was scraped from thin layer cellulose plates and extracted with 5% formic acid and 15% acetic acid. The extracted peptide was lyophilized and then hydrolyzed by heating at 110 °C for 6 h in 6 N HCl. The phosphoamino acid analysis was performed by the methods of Boyle et al. (36).

**Expression in HEK293 Cells—** The enhanced green fluorescent protein (EGFP) tagged NF-H expression vector was constructed from NF-H cDNA by cloning its EcoRI fragment into the EcoRI site of pEGFP-C1 (CLONTECH). The cloned plasmid was digested with BglII and NotI, and self-ligated to adjust the reading frame after blunt ending. The FLAG-tagged tail domain expression vector was constructed by cloning the BamHI-SalI fragment of the tail domain E. coli expression vector into HindIII-SalI sites of pCMV2-FLAG. The FLAG-tagged E-segment expression vector was constructed by cloning the EcoRI-SalI E-segment fragment of the E-segment E. coli expression vector into the EcoRI-SalI site of pCMV2-FLAG. The GSK3β and p35 expression plasmids were constructed by cloning the 5′ and 3′ flanking regions of cDNAs into pCMV5. The constitutively active MEK1 (CA-MEK) expression plasmid was a gift from Dr. E. Nishida (Kyoto University, Japan).

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 100 units/ml of penicillin and 0.1 mg/ml streptomycin. Transfection was performed using the Polyfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The transfected cells were cultured for 24 h and

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FIG. 1. Phosphorylation at Ser-493 and Ser-501 in the E-segment of NF-H prepared from rat spinal cords. A, amino acid sequence of the rat NF-H E-segment. The E-segment (bold) is located at the N terminus of the tail domain. Five CK-consensus phosphorylation sites are indicated by arrowheads, and two SP sequences are indicated by double arrowheads. Lysyl-endopeptidase digestion fragments are indicated by brackets underneath with predicted molecular masses. Amino acid sequence 425–507 (underlined) was the fragment used as substrate to purify the E-segment kinase. The dotted line indicates the amino acid region reported by Elhanany et al. (37) to contain no phosphorylation site. B, protein staining of NFs (lane 1) and dephosphorylated NFs (lane 2) separated on a 7.5% SDS-PAGE gel. NFs were prepared from rat spinal cords by gel filtration and dephosphorylated with E. coli alkaline phosphatase. NF-H, dephosphorylated NF-H, NF-M, dephosphorylated NF-M, NF-L, and E.coli alkaline phosphatase are indicated by H, dH, M, dM, L, and ALP, respectively. Molecular masses are indicated at the left of gel. C, mass spectrometric signals of phosphorylated (upper panel) and dephosphorylated (lower panel) NF-H excised from the gels by digestion with lysyl-endopeptidase. Molecular masses of 2836.50 and 3292.31 coincided with the predicted masses of 449–472 amino acid and 473–504 amino acid fragments, respectively, derived from the E-segment. Molecular masses of 3371.49 and 3451.18 corresponded to the predicted masses of the 473–504 amino acid fragment plus 80 and 160, respectively. D, two peptide fragments with molecular masses of 3371.49 and 3451.18 were analyzed for phosphorylated amino acid by the method of MS/MS. The Y-series signals were assigned to amino acid sequences using the MASCOT on-line search program. Ser-493 was phosphorylated in the peptide fragment with the molecular mass of 3371.49 (S + P, in upper panel), and Ser-493 and Ser-501 were phosphorylated in the fragment with molecular mass of 3451.18 (S + P, in lower panel).
Phosphorylation at Ser-493 of the NF-H E-segment by GSK3β

RESULTS

Ser-493 and Ser-501 in the E-segment of NF-H Were Phosphorylated in Rat Spinal Cords—The amino acid sequence of the rat NF-H E-segment (bold) is shown in Fig. 1A with possible phosphorylation sites for CKs (arrowheads) and proline-directed protein kinases (double arrowheads). Amino acid residues 417–448 of the E-segment (Fig. 1A, dot underline) were previously shown to be not phosphorylated in NF-H prepared from rat spinal cord (37). We focused our targets to the latter two peptides, amino acids 449–472 and 473–504. Because the predicted molecular masses of their lysyl-endopeptidase digestion fragments are appropriate sizes for mass analysis (Fig. 1A, brackets), mass spectrometry was used to analyze the in vivo phosphorylation state of the NF-H E-segment. NFs were prepared from rat spinal cord extract by Sepharose CL-4B gel filtration chromatography (Fig. 1B, lane 1). NF-H cut of SDS-PAGE gels was digested with lysyl-endopeptidase. The resulting peptides were subjected to a matrix-assisted laser desorption ionization-time of flight mass spectrometer. Three signals were detected at molecular masses of 2638.50, 3371.49, and 3451.18 (Fig. 1C, upper panel). The signal with molecular mass of 2836.50 corresponded to the predicted mass of fragment 449–472 of the E-segment (Fig. 1A), indicating that it was not phosphorylated, although there are three consensus phosphorylation motifs for CKs. The molecular masses of 3371.49 and 3451.18 matched with the predicted masses (3289.25) of fragment 473–504 (Fig. 1A) that bound one (+80) and two (+160) phosphates, respectively. The latter two signals disappeared, and instead a new signal at a mass of 3292.31 appeared when NF-H (Fig. 1B, lane 2) was dephosphorylated (Fig. 1C, lower panel), indicating that the peptide fragment of amino acids 473–504 was phosphorylated at one or two sites in rat spinal cords.

The two phosphorylation sites in fragment 473–504 were determined by the method of MS/MS using a quadrupole-time of flight mass spectrometer. The Y-series signals assigned by the MASCOT on-line service (www.matrixscience.com) are shown in Fig. 1D. Ser-493 and both Ser-493 and Ser-501 were phosphorylated in the peptide fragment with one phosphate (Fig. 1D, upper panel) and with two phosphates (Fig. 1D, lower panel), respectively.

Identification of an NF-H E-segment Kinase as GSK3β—An NF-H E-segment kinase was purified from rat brain extracts using the E-segment expressed in E. coli as a substrate. We chose the whole E-segment (amino acids 425–507, underlined in Fig. 1A), instead of a smaller fragment encompassing just Ser-493 and Ser-501, as substrate for the kinase purification to allow for possible phosphorylation at Ser/Thr residues other than Ser-493 and Ser-501 in the E-segment. The rat brain extract was first fractionated into DEAE-cellulose-unbound and -bound fractions. An E-segment kinase activity was detected only in the DEAE-cellulose-unbound fraction. The E-segment kinase was partially purified from the DEAE-cellulose-unbound fraction by column chromatography using SP-Sepharose, heparin-Sepharose, and Superose 6 gel filtration (Fig. 2A) as described under “Materials and Methods.” The kinase activity was detected as a single peak throughout all three columns. Because of possible phosphorylation sites for both CKs and proline-directed protein kinases in the E-segment, we estimated their contribution to phosphorylation of the E-segment at each purification step by the in-gel assay for CKs or by inhibition with olomoucine, butyrolactone I, and LiCl, for proline-directed protein kinases. The CK activity was not detected in any of the E-segment kinase fractions (data not shown). LiCl alone inhibited the kinase activity in each step. Fig. 2B shows inhibition of the E-segment kinase activity eluted from a Superose 6 gel filtration column by 20 mM LiCl, suggested it to be GSK3. This was further supported by co-elution of the kinase activity with anti-GSK3β antiserum. The kinase fraction (fraction 39) of Superose 6 gel filtration was added with nonimmune rabbit antiserum (lane 1) and an increased amount of anti-GSK3β rabbit antiserum (0 μl, lane 2; 0.3 μl, lane 3; 3.0 μl, lane 4), and immunoprecipitates were used for the E-segment kinase assay with [γ-32P]ATP. Phosphorylation of the E-segment is shown in B and C.

Inhibition of the E-segment kinase activity by LiCl. The E-segment kinase activity of fraction 39 was assayed in the presence of 20 mM NaCl (lane 2) or 20 mM LiCl (lane 3) using [γ-32P]ATP. The control kinase activity is shown in lane 1. C, immunoprecipitation of the E-segment kinase with anti-GSK3β antisemur. The kinase fraction (fraction 39) of Superose 6 gel filtration was added with nonimmune rabbit antiserum (lane 1) and an increased amount of anti-GSK3β rabbit antiserum (0 μl, lane 2; 0.3 μl, lane 3; 3.0 μl, lane 4), and immunoprecipitates were used for the E-segment kinase assay with [γ-32P]ATP. Phosphorylation of the E-segment is shown in B and C.
Phosphorylation at Ser-493 of the NF-H E-segment by GSK3β

There are two Ser residues, Ser-493 and Ser-501, in fragment 473–504. To determine which is phosphorylated by GSK3β, we generated mutants of the E-segment (S493A, S501A, and S493/501A) whose Ser-493 and Ser-501 was replaced with Ala and phosphorylated them with GSK3β. This result indicates that the phosphorylation site in the E-segment is Ser-493. At present, we do not know why both spots disappeared from a single mutation at Ser-493.

Ser-493 was the Preferred Phosphorylation Site in the Tail Domain of NF-H for GSK3β—GSK3 is reported to phosphorylate the KSP repeats in the NF-H tail region (22, 38). Ser-493 is in the SP sequence located in the E-segment, upstream of the KSP repeat region. We compared Ser-493 with the KSP repeats with respect to substrate preference for GSK3β. When the whole tail domain including both Ser-493 and the KSP repeat was used as a substrate, Ser-493 (spots 1 and 2) was phosphorylated earlier than the KSP repeat (several spots on line 3) (Fig. 4B, left panel). The signals for the KSP repeats became stronger at a plateau stage (Fig. 4B, right panel). This result suggests that Ser-493 in the E-segment is the better phosphorylation site than the KSP repeat in the NF-H tail domain for GSK3β.

There are two other classes of proline-directed protein kinases, mitogen-activated protein kinase family kinases and Cdk5 in neurons, which are also capable of phosphorylating KSP repeats. Comparing Ser-493 phosphorylation by GSK3β, with those by ERK2 and Cdk5, the E-segment was found to be a better substrate for GSK3β than ERK2 and Cdk5 (Fig. 5, upper panel). This was not because of the relative strength of the kinase activities used. When the whole tail domain was used as a substrate, ERK2 phosphorylated it most intensely,
Cdk5 moderately, and GSK3β marginally (Fig. 5, lower panel). These results suggested that Ser-493 in the E-segment is mainly phosphorylated by GSK3β and the KSP repeats mainly by ERK and Cdk5.

Ser-493 Was Phosphorylated by GSK3β in Cultured Cells—To verify that GSK3β is the Ser-493 kinase in cultured cells, we transfected 293 cells with the cDNAs of NF-H and GSK3β, and studied the phosphorylation of Ser-493. To detect specific phosphorylation at Ser-493 in NF-H, we produced a phosphorylation-dependent antibody (anti-pS493) against phosphorylated Ser-493. The specificity of the antibody was shown by its reaction with the E-segment phosphorylated by GSK3β in vitro but not with the unphosphorylated E-segment (Fig. 6A). Anti-pS493 antibody reacted with native phosphorylated NF-H isolated from spinal cords but did not react with dephosphorylated NF-H (Fig. 6B), confirming the result of mass analysis in Fig. 1. Phosphorylation of Ser-493 in the E-segment and full-length NF-H was shown by co-transfection with GSK3β into 293 cells (Fig. 6, C and D). The pS493 antibody did not react with the E-segment and NF-H expressed in 293 cells but reacted with them when co-transfected with GSK3β. The results indicate that GSK3β phosphorylates NF-H at Ser-493 under cellular conditions.

GSK3β phosphorylated at Ser-493 preferable to the KSP repeats (Fig. 4), and mitogen-activated protein kinase and Cdk5 were less active against the E-segment than GSK3β (Fig. 5). This was confirmed in transfected cultured cells. The NF-H tail domain was co-transfected with GSK3β, Cdk5 activator p35, or CA-MEK, and was analyzed by reactivity to pS493 antibody for phosphorylation at Ser-493 and by mobility shift on SDS-PAGE for phosphorylation of the KSP repeats. The tail domain shifted upward distinctly when co-transfected with p35, which activates endogenous Cdk5, and was a broad band when co-transfected with CA-MEK. This result is consistent with previous reports (21, 38, 39). On the other hand, when the tail domain was co-transfected with GSK3β, it did not show the mobility shift despite phosphorylation at Ser-493.

Association of GSK3β with NF Cytoskeletons—In Wnt signaling, GSK3β and its substrate protein β-catenin form the APC complex with axin and several other proteins, in which axin serves as a scaffolding protein to facilitate the phosphorylation of β-catenin by GSK3β (40). Therefore, further experiments were designed to determine whether NFs associate with GSK3β. The crude extract of rat spinal cords was fractionated by gel filtration on a Sepharose CL-4B column. NFs were eluted at void volume fractions where approximately half of GSK3β was detected despite the molecular mass of GSK3β being 47 kDa (Fig. 7A). This was not a nonspecific aggregate of GSK3β because GSK3β was eluted in low molecular weight fractions in the presence of 1 M NaCl (Fig. 7A). To confirm the binding to NFs, a void volume fraction containing both NFs and GSK3β was centrifuged and examined for sedimentation of GSK3β with the NFs. GSK3β was detected in the NF pellets (data not shown). Following addition of 1 M NaCl, GSK3β remained in the supernatant, indicating that GSK3β may associate with NF cytoskeletons via ionic interaction. Rebinding of GSK3β to NFs was examined by mixing NFs and GSK3β, which were once dissociated in the presence of 1 M NaCl. NFs in the void volume fraction and GSK3β in fraction 31 of Sepharose CL-4B in the presence of 1 M NaCl (Fig. 7A) were dialyzed against the Pipes buffer, mixed, and centrifuged at 100,000 × g for 30 min to pellet NFs. The supernatant and the pellet were analyzed for GSK3β by Western blotting. GSK3β alone remained in the supernatant, but when incubated with NFs approximately half of the GSK3β was sedimented (Fig. 7B). This sedimentation was
consensus sites in the NF-H E-segment were phosphorylated in rat spinal cord as shown here (Fig. 4B). The other two consensus phosphorylation motifs for CKs in amino acid residues 417–448 of the E-segment (Fig. 1A, dot underline) were previously shown to be not phosphorylated in NF-H prepared from rat spinal cord (37). Thus, in the case of NF-H, the E-segment is not, at least constitutively, phosphorylated by CKs.

However, the NF-H E-segment was in fact phosphorylated in spinal cords and phosphorylation sites were identified at Ser-493 and Ser-501 by mass spectrometric analysis. Ser-493 of most NF-H molecules is phosphorylated stoichiometrically in spinal cords. Phosphorylated Ser-493 was detected in fragment 473–504 bearing both one and two phosphates and its unphosphorylated form was not detected in native NFs. In vivo phosphorylation was confirmed by an in vitro phosphorylation experiment of native NF-H by GSK3β. Ser-493 of NF-H in native NFs was not phosphorylated by GSK3β unless it was dephosphorylated prior for phosphorylation (data not shown). Moreover, in vivo phosphorylation at Ser-493 was ascertained by Western blotting with the antibody raised against phosphorylated Ser-493 (anti-pS493). Ser-493 is the first instance of in vivo and in vitro phosphorylation in the E-segment of NF-H.

GSK3β is the Ser-493 kinase of rat NF-H. GSK3β was the only protein kinase purified as an E-segment kinase from the DEAE-cellulose-unbound fraction of the rat brain extracts. Although this purification was incomplete, we believe that GSK3β is the only kinase in our purified enzyme fractions responsible for phosphorylation of the E-segment because: (i) the co-fractionation of GSK3β with the E-segment kinase activity in each column chromatography; (ii) inhibition of the E-segment phosphorylation by LiCl, a GSK3 specific inhibitor; and (iii) immunoprecipitation of the E-segment kinase activity using anti-GSK3β antiserum.

GSK3β, purified as the E-segment kinase from rat brains, phosphorylated at Ser-493 in vitro and phosphorylated the site in HEK293 cells when transfected. We hypothesize that Ser-493 is also phosphorylated by GSK3β in spinal cords based on the following evidence: (i) GSK3β preferred Ser-493 to the KSP-repeated sequences for phosphorylation sites in the NF-H tail domain (Figs. 4 and 6E); (ii) Ser-493 was the better phosphorylation site for GSK3β than other proline-directed protein kinases, Cdk5/p35 and ERK (Figs. 5 and 6E); and (iii) NF cytoskeletons associated with GSK3β in the spinal cord extract (Fig. 7).

Ser-501, in addition to Ser-493, was phosphorylated in a portion of NF-H prepared from rat spinal cords. However, the site was not phosphorylated by GSK3β, the E-segment kinase isolated from rat brains. The fact that we did not detect any E-segment kinase activities other than GSK3β in purification steps from the DEAE-cellulose-unbound fraction may suggest that the Ser-501 kinase binds to the DEAE-cellulose in the first purification step. Ser-501 is in the SP sequence followed by Lys at the third C-terminal site. The SPXXK sequence is a consensus phosphorylation motif for Cdk5 (17, 18, 20) and also mitogen-activated protein kinase. Because Ala substitution of Ser-501 reduced the phosphorylation of the E-segment by Cdk5 and ERK (data not shown), we suggest that this site would be phosphorylated by Cdk5 and ERK. Because both Cdk5 and ERK bound to DEAE-cellulose in our column chromatographic conditions, Ser-501 kinases might not be detected in the DEAE-cellulose-unbound fraction. We had chosen the DEAE-cellulose-unbound fraction as a starting material for purification because the DEAE-cellulose-bound fraction did not display E-segment kinase activity when bound proteins were eluted at once with a high salt solution. However, in our recent experiments we have found E-segment kinase activities in fractions

**DISCUSSION**

In this study, we demonstrated phosphorylation of Ser-493 and Ser-501 in the E-segment of the NF-H tail domain from rat spinal cords, using mass spectrometric analysis. We partially purified the E-segment kinase from rat brain extracts and identified it as GSK3β. Purified GSK3β-phosphorylated Ser-493 in vitro and prefer Ser-493 to the KSP repeats for phosphorylation in the tail domain of NF-H. GSK3β also phosphorylated Ser-493 in HEK293 cells. GSK3β in the spinal cord extracts was shown to associate with NF cytoskeletons. Taken together, these results indicate that GSK3β is the physiological kinase for Ser-493 of rat NF-H.

The E-segments are a glutamic acid-rich region present in the C-terminal tail domain of all three NF-subunits (1, 3). Although the amino acid sequence of respective E-segments differs from each other, each E-segment in respective NF subunits is highly conserved among mammalian species (41). However, the role of the E-segment has not been elucidated, although it has been suggested to be important for filament assembly (27, 42). Considering that one of the particular properties of the tail domain of NF subunits is their phosphorylation, it was interesting to investigate the phosphorylation of the E-segment of different NF subunits. The E-segments of NF-L and NF-M have already been reported to be phosphorylated by CKs (29, 31, 32). However, none of the five Ser/Thr CKs

**FIG. 7. Association of GSK3β with NFs in the spinal cord extract.** A, co-elution of GSK3β with NFs in gel-filtration column chromatography. A rat spinal cord extract was fractionated by a Sepharose CL-4B gel filtration column in either a low salt solution (Buffer 1) or a high salt solution (Buffer + 1 M NaCl). Coomassie Brilliant Blue (CBB) staining of an SDS-PAGE gel in a low salt solution is shown in the top panel. Molecular weight standards are indicated at the right side and NF-H, NF-M, and NF-L are indicated by H, M, and L, respectively, at left side of the gel. Lower panels are Western blots of proteins eluted in the low salt and high salt buffer, respectively, using an anti-GSK3β antiserum. B, co-sedimentation of GSK3β with NFs. NFs (fraction 15) and GSK3β (fraction 31), which were once separated by gel filtration in a high salt solution in A, were dialyzed against the low salt solution. Western blots with anti-GSK3β antiserum are shown in lanes 1 and 2. GSK3β was mixed with the low salt buffer alone (lanes 3 and 5) or NFs (lanes 4 and 6), and then the mixtures were centrifuged at 100,000 × g for 30 min to pellet NFs. The supernatant (lanes 3 and 4) and the pellet (lanes 5 and 6) were analyzed for GSK3β by Western blot.

not observed in the presence of 0.75 M NaCl (data not shown). These results suggest an association of GSK3β with NFs.
Phosphorylation at Ser-493 of the NF-H E-segment by GSK3β

Phosphorylation of the NF-H tail domain by GSK3β has been somewhat controversial. Guidato et al. (38) showed phosphorylation of the KSP repeats in NF-H by GSK3α in transfected COS cells using several KSP phosphorylation-dependent antibodies. On the other hand, Sun et al. (21) failed to detect significant phosphorylation of the KSP repeats in NF-H by GSK3β in similar transfection experiments using SW13 cl.2 Vim- cells. Tau protein kinase I, identical to GSK3β, did not phosphorylate the peptides containing KSP sequences in vitro (18). The variance in these reports may be because of the different isoforms used, GSK3α and -β, although they have a very similar substrate specificity (43). However, it is more likely that the strength of GSK3 activity in transfected cells or in the reaction mixture determines the phosphorylation of the KSP repeats. We did not observe the phosphorylation-dependent mobility shift of the NF-H tail domain, such as those shown by Cdk5/p35 (co-transfection of p35) or ERK (co-transfection of CA-MEK), when it was co-transfected with GSK3β by Cdk5/p35 (co-transfection of p35) or ERK (co-transfection of CA-MEK), when it was co-transfected with GSK3β in 293 cells.

In our in vitro phosphorylation experiments, however, the KSP repeats were in fact phosphorylated by GSK3β, although their phosphorylation appeared to be a later event after Ser-493 phosphorylation (Fig. 4). The KSP repeats may be the secondary phosphorylation sites in NF-H for GSK3β.

The NF-H tail domain is a unique protein because it can be phosphorylated at different sites by three different proline-directed protein kinases of MAP kinases (ERK and stress-activated protein kinase), Cdk5, and GSK3β. The KSPXX and KSPXXK sequences in the KSP motif of the NF-H tail domain are phosphorylated by ERK and Cdk5, respectively (18, 21–23). Ser-493 in the SP sequence in the E-segment was phosphorylated by GSK3β. Considering that these protein kinases play important roles in a variety of neuronal activities, it is of great interest to know how their phosphorylation events are regulated and what roles their phosphorylation play. The well-known, but unresolved, question is the uneven distribution of the KSP phosphorylation in neurons: the unphosphorylated form in the cell body and dendrites and the phosphorylated form in the axons (3). Distribution of NF-H with phosphorylated Ser-493 is under investigation using pS493 antibody. The role of Ser-493 phosphorylation is also a question to be addressed in the future. Because the E-segment appears to be involved in filament formation (27, 42), phosphorylation in that region may also play a regulatory role in filament formation. Secondary structure prediction suggests that phosphorylation of Ser-493 in combination with following the Pro residue interrupts α-helix of the E-segment.

The E-segment of NF-L and NF-M are phosphorylated by Cks associated with NFs (29, 31, 32). GSK3β also appears to associate with NFs in the rat spinal cord extract. GSK3β was coeluted during gel-filtration column chromatography and co-sedimented by centrifugation with NFs, respectively. It is well known in undifferentiated proliferating cells that GSK3β binds to a large protein complex including axin as a scaffold protein and β-catenin as a substrate. This is assumed to be the mechanism to choose β-catenin as a target protein (40). Association of GSK3β with NF cytoskeletons may also increase effective phosphorylation of Ser-493 in axons. How GSK3β associates with NFs will be a question to be investigated.
In Vivo and in Vitro Phosphorylation at Ser-493 in the Glutamate (E)-segment of Neurofilament-H Subunit by Glycogen Synthase Kinase 3 β
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