Neurofilament-Associated Protein Phosphatase 2A: Its Possible Role in Preserving Neurofilaments in Filamentous States†

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Received November 11, 1994; Revised Manuscript Received March 2, 1995⊗

ABSTRACT: Neurofilament phosphatase (NF-phosphatase) activity, which dephosphorylates NF proteins phosphorylated by cyclic AMP-dependent protein kinase (A-kinase), was detected in NF fractions prepared from bovine spinal cords. This phosphatase was suggested to be associated with NFs by gel filtration and sedimentation analysis and was further demonstrated by dephosphorylation-dependent binding assay of NFs to microtubules. The NF-associated NF-phosphatase was identified as a type of protein phosphatase 2A (PP2A) by (i) its complete inhibition with 100 nM okadaic acid, at which concentration the purified type 1 protein phosphatase (PP1) was inhibited only 25%; (ii) the absence of effect of inhibitor-2, a specific inhibitor of PP1, on the NF-phosphatase activity; and (iii) the detection of 38-kDa catalytic and 65-kDa regulatory subunits of PP2A by immunoblotting. The NF-associated PP2A was partially solubilized from NFs by a high concentration of $MgSO_4$, and the solubilized PP2A was suggested by gel filtration to be a dimeric holoenzyme consisting of a 38-kDa catalytic and a 65-kDa regulatory subunit. Phosphorylated NF-L, which is assembly incompetent, was induced to assemble into filaments by dephosphorylation with PP2A. These results suggest a role of NF-associated PP2A in preserving filamentous forms of NF in neurons.

It is now clear that phosphorylation and dephosphorylation reactions reversibly modify the functions of many proteins. In contrast to phosphorylation reactions that have been extensively studied, especially with regard to signal transduction pathways (Hunter, 1987; Taylor et al., 1990; Fantl et al., 1993), relatively little has been known about the regulatory roles of protein phosphatases in many cellular processes until recently (Cohen & Cohen, 1989). This was partly due to the broad substrate specificity of protein phosphatases and no apparent dynamic changes of their activity dependent on the cell cycle, growth, or differentiation, in addition to difficulties in experimental techniques (Cohen, 1989). However, recently they have been shown to be involved in many protein functions. For example, some protein phosphatases are reported to participate in promotion or suppression of cell cycle progression (Fernandez et al., 1992; Walker et al., 1992; Clark et al., 1993), and their substrate specificities were found to be altered by bound regulatory subunits (Sola et al., 1991; Agostinis et al., 1992; Ferrigno et al., 1993) or in association with specific subcellular compartments (Hubbard & Cohen, 1993).

Neurofilament (NF),[1] a major intermediate filament (IF) expressed in most neurons, is one such protein whose properties are regulated by phosphorylation (Nixon & Shea, 1992; Liem, 1993). NF is composed of three subunit proteins with apparent molecular masses of 200 (NF-H), 160 (NF-M), and 70 kDa (NF-L) on SDS−PAGE. Each NF subunit protein consists of three domains, an amino-terminal head, an α-helix-rich central, and a carboxy-terminal tail (Steinert & Roop, 1988). A number of experiments have suggested the involvement of the head and central domains in filament formation (Nixon & Shea, 1992; Liem, 1993). Phosphorylation of the head domain by cyclic AMP-dependent protein kinase (A-kinase) or protein kinase C (C-kinase) depolymerizes filamentous NF-L into an oligomeric form, probably an octamer, and inhibits polymerization of oligomeric NF-L (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990). Phosphorylation of all three subunits of native NFs with A-kinase resulted in fragmentation of filaments *in vitro* (Hisanaga et al., 1994). Although the A-kinase phosphorylation site of NF proteins was shown to be phosphorylated *in vivo* in rat optic nerve axons (Sihag & Nixon, 1991), most NF proteins are present as assembled forms in detergent-insoluble fractions (Morris & Lasek, 1982; Nixon et al., 1989), suggesting that NF-phosphatase is required to keep the head domains in the dephosphorylated state. Treatment with okadaic acid, an inhibitor of protein phosphatases 1 and 2A, causes disruption of the NF network and results in solubilization of NF proteins in rat primary cultured neurons

† This work was supported by grants from the Kato Memorial Foundation and Kanagawa Science Academy (to S.H.), a Grant-in-Aid for Scientific Research on Priority Areas No. 04268104 (to S.H.), a grant from the Asahi Glass Science Foundation (to T.K.), and Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (to T.K.).

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1995.

[1] Abbreviations: PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; PP2C, protein phosphatase 2C; A-kinase, catalytic subunit of cyclic AMP-dependent protein kinase; C-kinase, protein kinase C; NF, neurofilament; IF, intermediate filament.

0006-2960/95/0434-7376$09.00/0

(Sacher et al., 1992, 1994). NF proteins phosphorylated *in vitro* by A-kinase were better substrates for the catalytic subunit of protein phosphatase 2A (PP2A) than for protein phosphatase 2B (PP2B), protein phosphatase 2C (PP2C), and the catalytic subunit of protein phosphatase 1 (PP1) (Hisanaga et al., 1993b). NF-L phosphorylated *in situ* was dephosphorylated by the catalytic subunit of PP2A but not by PP1 (Sacher et al., 1994). Although these results suggest the involvement of PP2A in dephosphorylation at the head domains of NF proteins, it is unknown how protein phosphatase regulates the polymerization of NFs *in vivo*.

In a previous study (Hisanaga et al., 1994), we used NF protein phosphorylated with A-kinase as substrate and suggested the presence of phosphatase activity in crude NF preparations of bovine spinal cords. In this paper, we describe the association of NF-phosphatase with NFs and its identification as a type of protein phosphatase 2A, composed of a 38-kDa catalytic subunit and a 65-kDa A regulatory subunit (RP65). We also suggest its role in preserving the filamentous state of NF.

MATERIALS AND METHODS

Materials. Rabbit anti-rat PP2A 38-kDa catalytic subunit serum was raised against the C-terminal 25 oligopeptide (Shima et al., 1993). The anti-65-kDa A regulatory subunit (RP65) antibody used was that described by Hendrix et al. (1993). Okadaic acid was purchased from Wako Chemicals (Osaka, Japan).

Protein Phosphatases and Protein Kinase. The catalytic subunits of PP1 and PP2A prepared from skeletal muscle were provided by Dr. Mitsuo Ikebe (Case Western Reserve University, Cleveland). Inhibitor-2 was prepared from rabbit skeletal muscle by the method of Cohen et al. (1988a). The catalytic subunit of A-kinase was prepared from bovine heart by the method of Beavo et al. (1974).

Preparation of NF Proteins. NFs were prepared from bovine spinal cord extract by gel filtration (Hisanaga & Hirokawa, 1988). Bovine spinal cord was homogenized with an equal volume of PEM (0.1 M Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL leupeptin, and 0.5 mM dithiothreitol (DTT). The homogenate was centrifuged at 28000*g* for 30 min, and the supernatant fraction was used as a crude extract. The crude extract (0.5−1 mL) was applied to a Sepharose CL-4B gel filtration column (1.5 × 45 cm) and eluted with 20 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM EDTA, and 10% glycerol. NFs were eluted in void volume fractions. NFs were precipitated by centrifugation at 100000*g* for 90 min. In some experiments, buffers containing 0.01−0.1% Tween-20 or 1% Triton X-100 were used for preparation of NF.

Separation of NFs by Sucrose Density Gradient Centrifugation. NFs were fractionated by sucrose density gradient centrifugation (Mori et al., 1979). The NF fractions prepared by gel filtration were layered on top of a sucrose density gradient (0.5 M/1.0 M/2.2 M; each dissolved in 0.15 M NaCl, 10 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 0.01% Tween 20) and were then centrifuged at 200000*g* for 75 min at 4 °C. NFs were recovered at the interface between the 1.0 and 2.2 M sucrose layers.

Dephosphorylation of NFs with Alkaline Phosphatase and Their Cosedimentation with Microtubules. NFs (1 mg/mL), which were prepared by gel filtration of Sepharose CL-4B in 0.1 M NaCl, 20 mM Tris-HCl, pH 8.5, 1 μg/mL leupeptin, 1 mM DTT, and 0.2 mM PMSF, were dephosphorylated with *Escherichia coli* alkaline phosphatase (Wako, Osaka, Japan) at 35 °C for 12 h. At the end of incubation, NFs were separated from alkaline phosphatase by gel filtration in 0.15 M NaCl, 10 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 0.01% Tween 20, 1 μg/mL leupeptin, 1 mM DTT, and 0.2 mM PMSF, and the void volume fractions were used as the dephosphorylated NFs.

Microtubule protein was prepared from porcine brains by two cycles of temperature-dependent polymerization/depolymerization (Shelanski et al., 1973). Tubulin was purified from microtubules by phosphocellulose column chromatography (Weingarten et al., 1975).

Binding of NF proteins to microtubules was assayed by cosedimentation as described previously (Hisanaga & Hirokawa, 1990). Either the dephosphorylated or phosphorylated forms of NFs (0.6 mg/mL) were incubated with microtubules (1.2 mg/mL) in the presence of 20 μM taxol and 0.5 mM GTP for 30 min at 35 °C in 10 mM Pipes, pH 6.8, 1 mM EGTA, and 1 mM $MgCl_2$. After centrifugation at 7300*g* for 10 min at 30 °C, the supernatant fractions and pellets were analyzed by 7.5% SDS−PAGE and by blotting with antibody against PP2A catalytic subunit.

Preparation of ^{32}P-Labeled Substrate. About 0.2 mg/mL NF-L or NF-M was phosphorylated using [γ-^{32}P]ATP by treatment with A-kinase at 30 °C for 1 h. The reaction was stopped by the addition of ammonium sulfate to 75% saturation, and the phosphorylated NF-L or NF-M was precipitated by centrifugation at 10000*g* for 30 min. The pellet was washed twice with 75% saturated ammonium sulfate solution; suspended in 5 mM Tris-HCl, pH 8.5, containing 8 M urea; dialyzed against 5 mM Tris-HCl, pH 8.5, containing 0.5 mM EGTA and 0.1 mM EDTA at 4 °C; and used as a substrate for dephosphorylation.

Phosphorylase *a* was prepared by phosphorylation of phosphorylase *b* (Sigma) by phosphorylase kinase (Sigma) according to the method described by Cohen et al. (1988b).

Dephosphorylation Reaction. The dephosphorylation reaction was performed using about 30 μg/mL ^{32}P-labeled substrate in 20 mM Tris-HCl, pH 7.5, at 30 °C. The reaction was stopped either by addition of SDS-sample buffer or by spotting on Whatman P81-cellulose filter paper. Radioactivities remaining in substrate proteins were counted by Cerenkov radiation directly or after SDS−PAGE.

Electron Microscopic Observation of NFs. Phosphorylated or dephosphorylated NF-L (0.2 mg/mL) was assembled by dialysis against 10 mM Pipes, pH 6.8, containing 0.15 M NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, and 0.1 mM EDTA at 35 °C for 3 h. A drop of suspension containing filaments, reconstructed from NF-L, was applied to a Formvar-coated grid that had been subjected to glow discharge. After 30 s, the liquid was removed with filter paper, and NF-L filaments were stained negatively by drops of 1% uranyl acetate. The grids were examined in a 1200EX electron microscope (JOEL, Tokyo, Japan) operated at 80 kV.

Gel Electrophoresis and Immunoblotting. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out using an acrylamide concentration of 7.5% for separation of NF proteins and of 12.5% for separation of protein phosphatase. Proteins were visualized with Coomassie Brilliant Blue.

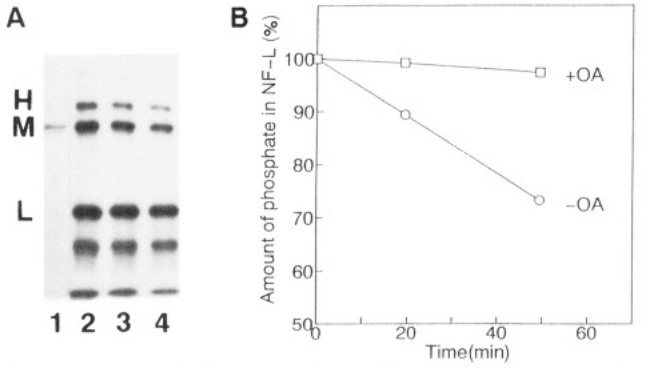

FIGURE 1: Autodephosphorylation of NF proteins in crude NF fractions phosphorylated by A-kinase. (A) Native NFs (0.2 mg/mL) prepared from bovine spinal cord by gel filtration were incubated with A-kinase in the presence of [γ-^{32}P]ATP at 35 °C. Aliquots were withdrawn at 0 (lane 1), 20 (lane 2), 40 (lane 3), and 120 min (lane 4), and the phosphorylation reaction was stopped by the addition of SDS−PAGE sample buffer and autoradiographed after SDS−PAGE. NF-H, NF-M, and NF-L are indicated by H, M, and L, respectively. (B) Native NFs were incubated with A-kinase at 35 °C for 10 min. The reaction was stopped by the addition of 5 mM EDTA, and phosphate bound to NF-L was measured after further incubation at 35 °C for 20 and 50 min by Cerenkov radiation of the NF-L band excised from SDS−PAGE gel (circles). Inhibition of dephosphorylation of NF-L by okadaic acid (1 μM) is indicated by squares.

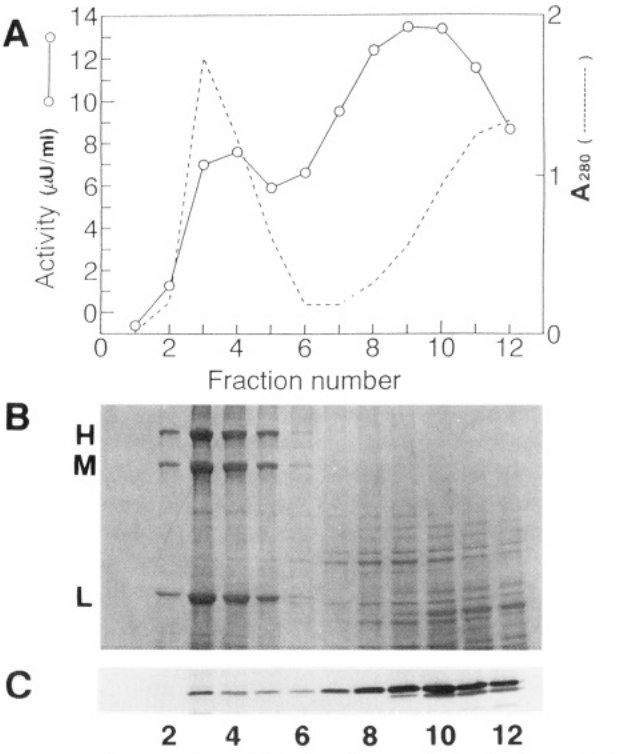

FIGURE 2: Fractionation of NF-phosphatase in the bovine spinal cord extract by Sepharose CL-4B gel filtration. The bovine spinal cord extract was applied to Sepharose CL-4B (15 × 45 cm), and the NF-phosphatase activity in each fractions was measured using NF-L phosphorylated by A-kinase as a substrate. The NF-phosphatase activity (○) and absorbance at 280 nm (dotted line) are indicated in panel A. (B) 7.5% SDS−PAGE of each fraction. NF-H, NF-M, and NF-L are indicated by H, M, and L, respectively. (C) Immunoblot of each fraction with anti-38-kDa PP2A catalytic subunit. The 38-kDa PP2A catalytic subunit was detected as a small peak in void volume fractions in addition to a major peak in the lower molecular weight region.

Immunoblot analyses were performed with the anti-PP2A 38-kDa catalytic subunit, which recognizes both α and β isotypes, and the anti-65-kDa regulatory subunit, which also recognizes both α and β isotypes using an alkaline phosphatase conjugated anti-rabbit secondary antibody to detect bound antibodies.

Protein Concentration Determination. Protein concentration was determined by the procedure of Bradford (1976) with bovine serum albumin as a standard.

RESULTS

Cofractionation of NF-Phosphatase with NFs. When native NFs, which were prepared from bovine spinal cord by gel filtration, were phosphorylated by A-kinase, the amount of phosphate molecules incorporated into each NF protein (NF-L, NF-M, and NF-H) decreased after a peak at about 20 min (Figure 1A). This result suggests the presence of protein phosphatase(s) that remove(s) phosphates introduced by A-kinase in the native NF preparations. To estimate the NF phosphatase activity more clearly, we stopped the phosphorylation reaction after 10 min by the addition of EDTA to the reaction mixtures and measured the amounts of phosphate bound to each NF subunit for 50 min. The result for NF-L is shown in Figure 1B (circles). About 25% of the phosphate incorporated into NF-L was removed within 50 min. This decrease was shown to be due to dephosphorylation, but not due to protein degradation, by the fact that the addition of okadaic acid, a potent inhibitor of PP1, PP2A, and protein phosphatase 4 (PP4), to the reaction mixture suppressed the decrease of NF-L-bound phosphates (Figure 1B, squares). These results suggest that NF-phosphatase(s) present in the NF preparations is (are) okadaic acid-sensitive and Mg^{2+}-independent.

Native NFs were prepared from bovine spinal cord by only one step of gel filtration column chromatography, although NF triplet proteins were the major proteins in these fractions (see Figure 2B, lanes 2−5). To determine whether the NF-phosphatase present in the native NF preparations was simply contaminating soluble protein phosphatase(s) or not, the NF-phosphatase activity in each fraction from a Sepharose CL-4B gel filtration column was measured using NF-L or NF-M phosphorylated by A-kinase as an exogenous substrate. An elution profile is shown in Figure 2A. The NF-phosphatase activity was detected in two peaks, one in the void volume, where NFs were eluted (Figure 2B), and the other in lower molecular weight fractions. The major activity eluted in the low molecular weight region might represent soluble forms of protein phosphatases. To determine if the NF-phosphatase activity eluted in the void volume could be associated with NFs, we rechromatographed the peak fraction in the void volume (fraction 3 in Figure 2) on a Sepharose CL-4B column. Most of the NF-phosphatase activity was again detected in void volume fractions together with NF proteins (Figure 3A,B). As protein phosphatase itself is unlikely to form large particles such as those excluded from Sepharose CL-4B beads, so this result suggests its association with very large structures such as NF.

Cofractionation of the NF phosphatase with NFs was next examined by sedimentation methods. First, NFs eluted in void volume fractions were sedimented by centrifugation at 100000*g* for 90 min, and the NF-phosphatase activity in the pellet was measured. Most NF-phosphatase activity was detected in the pellet fraction (data not shown, see Figure 8, lanes 1 and 2 for reference). However, the above two

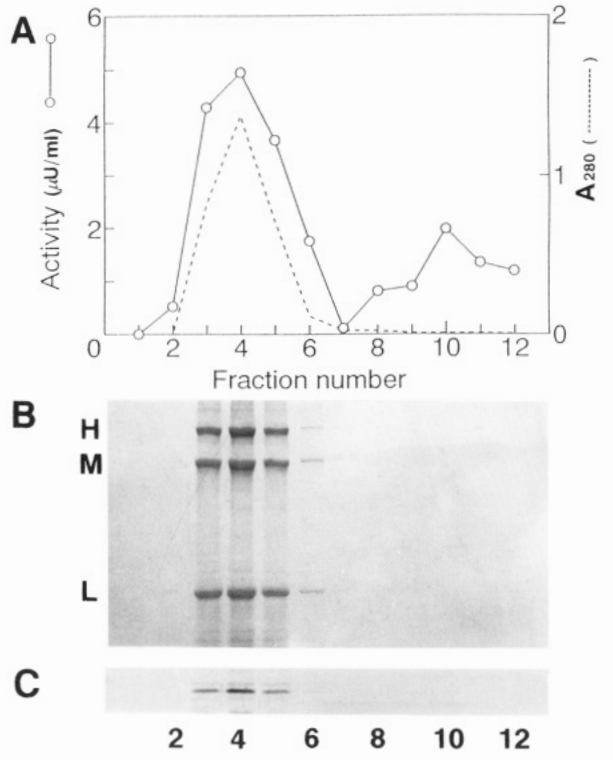

FIGURE 3: Rechromatography of the peak fraction of NF proteins and NF-phosphatase in the void volume on Sepharose CL-4B. Fraction 3 in Figure 2 was rechromatographed on a Sepharose CL-4B gel filtration column (0.5 × 24 cm). The elution profiles of proteins (dotted line) and NF-phosphatase activity (○), SDS−PAGE of each fraction, and immunoblotting with anti-38-kDa PP2A catalytic subunit are shown in panels A−C, respectively. NF-H, NF-M, and NF-L are indicated by H, M, and L, respectively.

methods could not discriminate the association of the NF-phosphatase with NFs from the association with particulate materials larger than NFs. Therefore, we subjected NFs to sucrose density stepwise gradient (0.5 M/1.0 M/2.2 M) centrifugation at 200000*g* for 75 min, which was previously used for the purification of NFs from rat peripheral nerves (Mori & Kurokawa, 1979). NFs were recovered at the interface between 1.0 and 2.2 M sucrose, and the NF-phosphatase activity was also detected in the interface fraction (Figure 4A), suggesting that some of the NF-phosphatase associates with NFs.

For exclusion of the possibility that NF-phosphatase was associated with membranes, the above experiments, gel filtration, and sedimentation were performed in the presence of a detergent (0.01−0.1% Tween-20 or 1% Triton X-100). The results were the same as before; that is, the NF-phosphatase activity was coeluted with NF proteins on gel filtration and coprecipitated with them (data not shown).

NF-Associated NF-Phosphatase Is a Type of PP2A. Protein phosphatases are classified into four types according to their requirement of divalent cations for the phosphatase activity, and to their inhibition by inhibitor-1 or -2 and okadaic acid (Ingebritsen & Cohen, 1983; Cohen, 1989). Since each of the four classes of purified catalytic subunit of protein phosphatases (PP1, PP2A, PP2B, and PP2C) is capable of dephosphorylating NF protein phosphorylated by A-kinase *in vitro* (Hisanaga et al., 1993b), the problem was what type of protein phosphatase(s) was (were) associated with native NFs. As described above (Figure 1B), the NF-phosphatase activity was sensitive to okadaic acid and

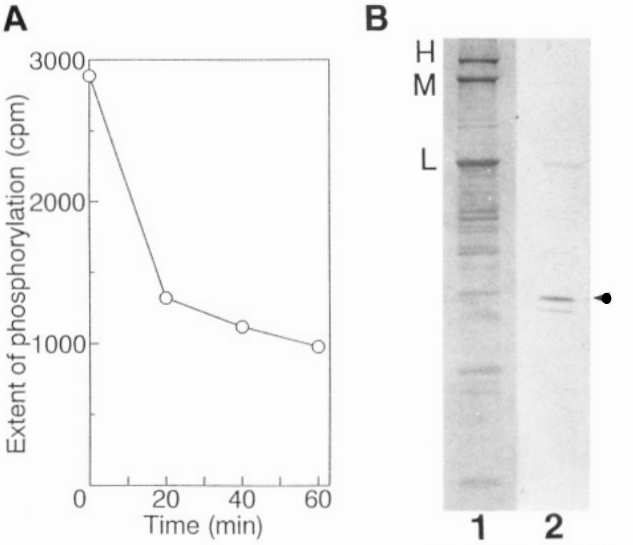

FIGURE 4: Detection of the NF-phosphatase in NFs isolated by sucrose density gradient centrifugation. NFs prepared by Sepharose CL-4B gel filtration were layered on top of a sucrose density gradient and centrifuged as described in Materials and Methods. NFs recovered at the interface between the 1.0 and 2.2 M sucrose were subjected to the NF-phosphatase assay using NF-L phosphorylated by A-kinase as a substrate (A), 12.5% SDS−PAGE (B, lane 1), and immunoblotting with anti-38-kDa PP2A catalytic subunit (B, lane 2, arrowhead). NF-H, NF-M, and NF-L are indicated by H, M, and L, respectively.

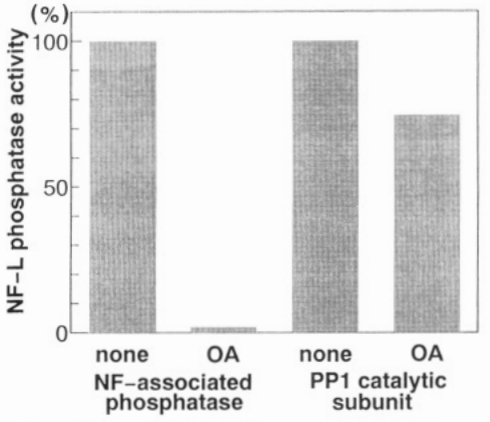

FIGURE 5: Inhibition of NF-phosphatase activity in NF preparations by okadaic acid. NF-phosphatase activity in the NF fractions was assayed using NF-L phosphorylated by A-kinase in the presence and absence of 100 nM okadaic acid. As a reference, the NF-L phosphatase activity of the PP1 catalytic subunit purified from skeletal muscle was measured in parallel with the same concentration of okadaic acid. Bars represent relative activity as compared with the activity in the absence of okadaic acid (100%).

insensitive to chelation of Mg^{2+} and Ca^{2+} (data not shown), suggesting it is not PP2B or PP2C. To determine if the NF-phosphatase was PP1, PP2A, or some other form, we first used okadaic acid because PP2A is much more sensitive than PP1 to inhibition by okadaic acid (Cohen et al., 1989). When assayed in the presence of 100 nM okadaic acid, NF-associated NF-phosphatase activity was almost completely inhibited, while only 25% inhibition of purified PP1 was observed (Figure 5).

Another common method to distinguish between PP1 and PP2A is the use of inhibitor-2, a specific inhibitor of PP1. The NF-associated NF-phosphatase was not inhibited in the presence of inhibitor-2 at concentrations at which most of the activity of purified PP1 was inhibited (Figure 6). These

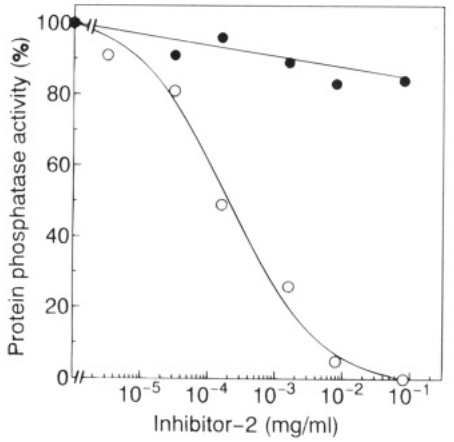

FIGURE 6: Inhibition of NF-phosphatase activity in the NF fraction by inhibitor-2. NF-phosphatase activity in the NF fractions (○) and phosphorylase activity of PP1 (●) were measured as functions of the concentration of inhibitor-2. The NF-associated NF-phosphatase activity was not affected in conditions that inhibited more than 95% of the PP1 activity.

results suggest that the activity of NF-associated NF-phosphatase was mainly that of PP2A.

This was confirmed by immunoblotting with the anti-catalytic subunit of PP2A. Figures 2C, 3C and 4B show the presence of an immunoreactive 38-kDa band with the same electrophoretic mobility as that of the catalytic subunit of PP2A purified from smooth muscle. The 38-kDa band was coeluted with NFs on gel filtration and cofractionated with NFs on sucrose density gradient centrifugation.

PP2A Showed Copartitioning with NFs in a Dephosphorylation-Dependent Association with Microtubules. We confirmed here the association of PP2A with NFs by utilizing a property of dephosphorylation-dependent association of NFs with microtubules (Hisanaga & Hirokawa, 1990). Because the NF-phosphatase activity in NF fractions was reduced, for unknown reasons, when NFs were brought to alkaline condition for dephosphorylation with *E. coli* alkaline phosphatase, we used the immunoblot with anti-PP2A catalytic subunit for its detection in this experiment. The native, phosphorylated form of NFs did not bind to microtubules, which were pelleted by a low centrifugation force of 7300*g* for 10 min, and remained in the supernatant fraction (Figure 7A, lanes 1 and 2). On the other hand, dephosphorylated NFs cosedimented with microtubules under the same centrifugation condition (Figure 7A, lanes 3 and 4). Their blotting with the anti-PP2A catalytic subunit shows copartitioning of PP2A with NFs; PP2A remained in the supernatant fraction together with the unbound, phosphorylated form of NFs and pelleted with dephosphorylated NFs (Figure 7B). Because the dephosphorylation-dependent binding to microtubules is a characteristic of NF-H (Hisanaga & Hirokawa, 1990), a simple interpretation of this result is the association of PP2A with NFs. Collectively, we conclude that most PP2A detected in the NF preparations associates with NFs, although we cannot completely exclude a possibility that a part of them binds to cellular components similar in size to or larger than NFs.

NF-Associated PP2A Contains the 65-kDa A Regulatory Subunit. Ethanol treatment, which is known to dissociate the free catalytic subunits from holoenzymes (Cohen et al., 1988a), released the 38-kDa catalytic subunit of PP2A from NFs (data not shown). However, to determine how PP2A

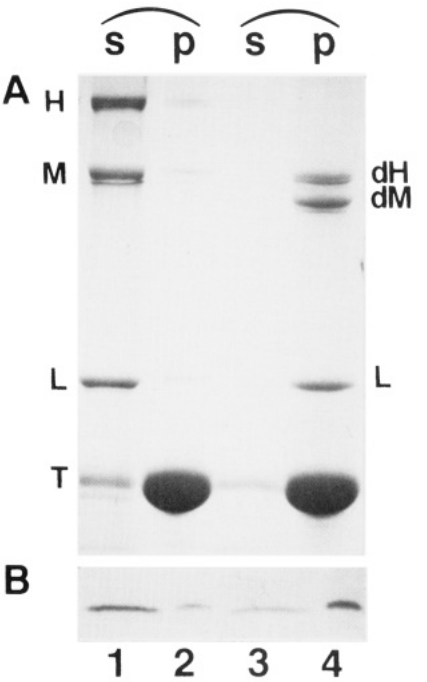

FIGURE 7: Dephosphorylation-dependent cosedimentation of NFs and associated PP2A with microtubules. Microtubules were polymerized from phosphocellulose-purified tubulin with the aid of 20 μM taxol. Phosphorylated and dephosphorylated NFs were respectively incubated with microtubules at 35 °C for 30 min and centrifuged at 7300*g* for 10 min. The supernatant fractions (lanes 1 and 3) and the pellets (lanes 2 and 4) were subjected to 7.5% SDS−PAGE (A) and immunoblotted with anti-38-kDa PP2A catalytic subunit (B). NF-H, NF-M, NF-L, and tubulin are indicated by H, M, L, and T, respectively, in lane 1. Dephosphorylated NF-H and NF-M are indicated in lane 4 by dH and dM.

is associated with NFs, it was necessary to dissociate NF-associated PP2A from NFs while preserving its subunit structure as far as possible. High salt solution (0.6 M NaCl) did not release PP2A from NFs (Figure 8A, lanes 3 and 4), suggesting that the binding was fairly strong. Therefore, we next used a high concentration of $MgSO_4$, which is effective for solubilizing PP1 associated with myofibrils (Nomura et al., 1992). A portion of PP2A was detected in the supernatant fraction when NFs were centrifuged in the presence of 0.3 M $MgSO_4$ (Figure 8A, lanes 5 and 6), and a further increase in $MgSO_4$ concentration to 0.6 M increased the amount of PP2A solubilized (Figure 8A, lanes 7 and 8). On the other hand, NF proteins were precipitated by centrifugation even in the presence of high concentrations of $MgSO_4$, confirming the solubilization of PP2A. The NF-associated PP2A was also partially solubilized by treatment with 2 M LiBr (data not shown) (Alessi et al., 1992; Dent et al., 1992).

In most tissues, PP2A is a heterotrimer composed of a 38-kDa catalytic subunit, a 65-kDa A regulatory subunit (RP65), and a B regulatory subunit with a molecular mass of 54−72 kDa or a heterodimer of the catalytic and 65-kDa regulatory subunit (Usui et al., 1988; Shenolikar & Nairn, 1991; Hendrix et al., 1993). Since the 65-kDa regulatory subunit is known to bind tightly to the catalytic subunit, we examined whether the NF-associated PP2A contained the 65-kDa regulatory subunit by immunoblotting. Immunoblotting with an antibody against the 65-kDa regulatory subunit indicated its existence in the NF fractions (Figure 8B). The 65-kDa immunoreactive protein showed the same behavior as the 38-kDa catalytic subunit on centrifugation or extraction

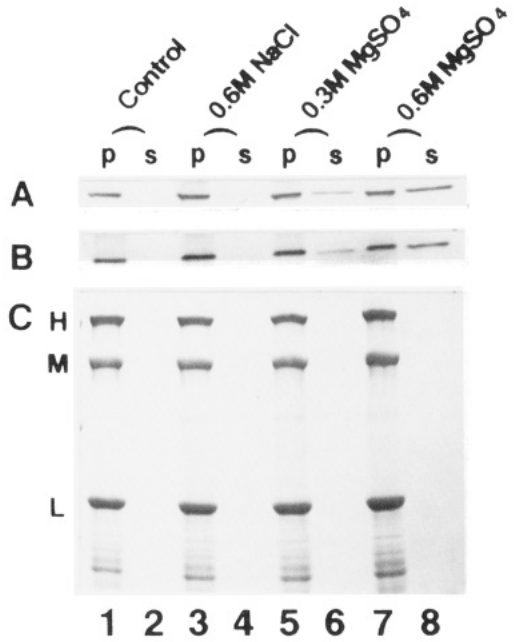

FIGURE 8: Dissociation of the NF-associated PP2A from NFs and identification of the 65-kDa regulatory subunit as its component. NFs fractionated by Sepharose CL-4B gel filtration were precipitated by centrifugation at 100000*g* for 90 min in the absence of additions (lanes 1 and 2) and in the presence of 0.6 M NaCl (lanes 3 and 4), 0.3 M $MgSO_4$ (lanes 5 and 6), or 0.6 M $MgSO_4$ (lanes 7 and 8). The precipitate (p) and the supernatant (s) were subjected to SDS−PAGE (C) and immunoblotting with anti-38-kDa catalytic subunit (A) and 65-kDa regulatory subunit (B) antibodies. The 65-kDa regulatory subunit showed the same behavior as the PP2A catalytic subunit on either NaCl or $MgSO_4$ treatment, suggesting complex formation with the 38-kDa catalytic subunit. NF-H, NF-M, and NF-L are indicated by H, M, and L, respectively.

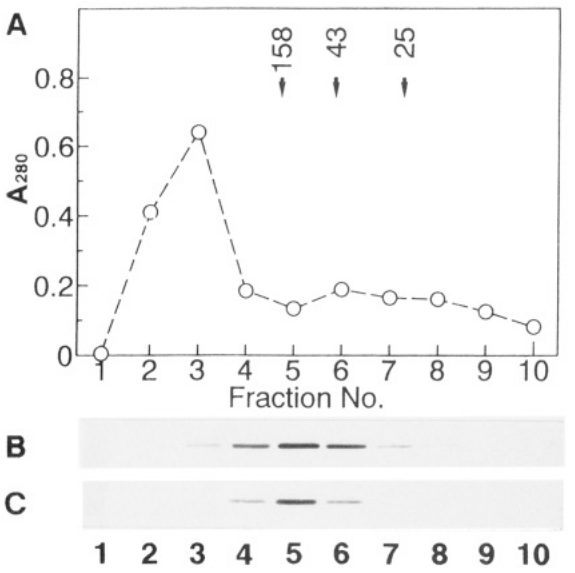

FIGURE 9: Gel filtration on Superose 12 of PP2A released with $MgSO_4$ from NFs. Eluted PP2A was detected by immunoblotting with anti-38-kDa (B) and anti-65-kDa (C) antibodies. The absorbance at 280 nm and elution of molecular mass markers are indicated in panel A. The two subunits were eluted together at a molecular mass of about 100-kDa, suggesting 1:1 heterodimer formation.

Table 1: Phosphatase Activity of NF-Associated Phosphatase, Catalytic Subunit of PP2A and PP1 toward NF-L and Phosphorylase *a*

substrate	NF-Pase[a]	PP2A[b]	PP1[c]
NF-L	1231[d]	1106	419
phosphorylase *a*	290	337	480

[a] The NF-associated phosphatase. [b] The catalytic subunit of PP2A purified from skeletal muscle. [c] The catalytic subunit of PP1 purified from skeletal muscle. [d] Activity is expressed by the amount of phosphate (cpm) released for 10-min incubation with each protein phosphatase.

with high concentrations of $MgSO_4$. PP2A extracted with $MgSO_4$ was gel-filtered on Superose 12 (Pharmacia) to estimate its molecular weight. The elution profile is shown in Figure 9. The catalytic subunit and the 65-kDa regulatory subunit were coeluted at a position corresponding to a molecular mass of about 100 kDa, suggesting that the catalytic and 65-kDa regulatory subunit formed a 1:1 complex.

PP2A Induces Assembly of the Assembly-Incompetent, Phosphorylated Form of NF-L by Dephosphorylation. The effect of dephosphorylation by PP2A on the filamentous state of NF was studied *in vitro*. In contrast to native NFs, which were only fragmented by phosphorylation with A-kinase, NF-L is disassembled completely into oligomeric forms (Hisanaga et al., 1990, 1994). Therefore, NF-L was used to study the effect of dephosphorylation on the filamentous state of NF. We also used a purified catalytic subunit of PP2A from skeletal muscle instead of NF-associated PP2A, which has not yet been purified, because the NF-associated PP2A and the purified catalytic subunit of PP2A showed similar substrate specificity toward NF proteins and a well-characterized phosphatase substrate, phosphorylase *a*. They had higher phosphatase activity toward NF proteins than toward phosphorylase *a*. This is in contrast to the catalytic subunit of PP1, which shows preference for phosphorylase *a* over NF proteins (Table 1).

A negative staining electron micrograph of reassembled NF-L is shown in Figure 10A. When reassembled NF-L was phosphorylated by A-kinase, NF-L disassembled into oligomeric forms (Figure 10B) as reported previously (Hisanaga et al., 1990; Nakamura et al., 1990). Figure 10D shows the time course of dephosphorylation of phosphorylated NF-L. The electrophoretic mobility shift of NF-L associated with phosphorylation and dephosphorylation is shown in the inset of Figure 10D. The filamentous state was examined by negative staining, after more than 90% of the phosphate incorporated into NF-L by A-kinase had been removed by dephosphorylation with the catalytic subunit of PP2A. Many filaments were observed in each sample of dephosphorylated NF-L (Figure 10C), indicating that dephosphorylation induced reassembly of NF-L.

DISCUSSION

We reported in this paper that the NF-phosphatase associated with NFs is a type of PP2A composed of, at least, the 38-kDa catalytic and the 65-kDa A regulatory subunits (RP65). Association of the NF-phosphatase with NFs was demonstrated by their coelution on Sepharose CL-4B gel filtration, cosedimentation with NFs on sucrose density gradient centrifugation, and copartitioning with NFs in a dephosphorylation-dependent binding assay to microtubules. The NF-phosphatase was identified as PP2A by inhibition experiments with okadaic acid and inhibitor-2 and by immunoblotting with antibodies against the PP2A 38-kDa catalytic and 65-kDa regulatory subunits. The finding that PP2A induced the reassembly of phosphorylated NF-L by

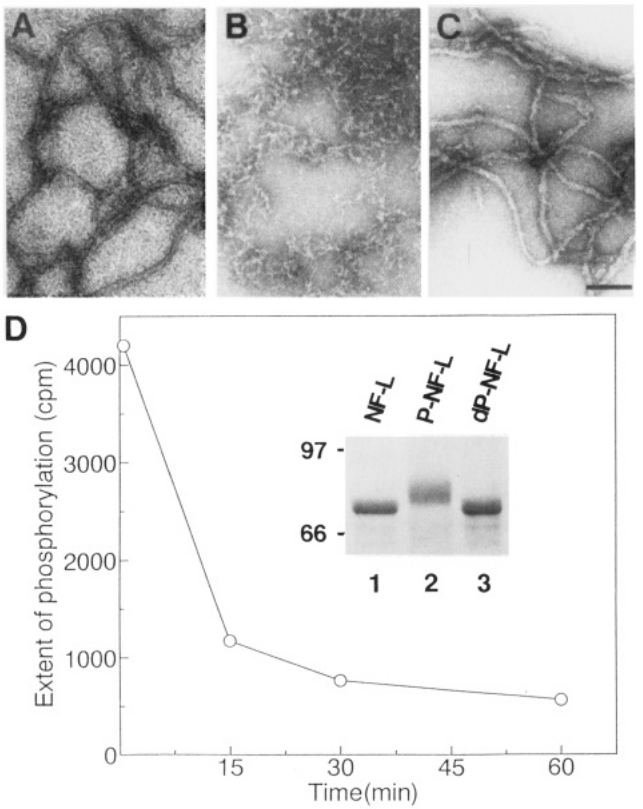

FIGURE 10: Effect of phosphorylation by A-kinase and dephosphorylation by PP2A on the filamentous state of NF-L. (A−C) Negative staining electron micrographs showing filamentous states of NF-L: (A) reassembled NF-L; (B) NF-L disassembled by phosphorylation with A-kinase; (C) dephosphorylated NF-L with PP2A showing filament formation. The bar represents 100 nm. (D) Time course of dephosphorylation of NF-L phosphorylated with A-kinase by a purified catalytic subunit of PP2A. The electron micrographs shown in panels B and C were obtained after incubation with PP2A for 0 and 120 min, respectively. The inset in panel D shows the electrophoretic mobility shift of NF-L according to phosphorylation and dephosphorylation. Lane 1, isolated NF-L; lane 2, NF-L phosphorylated by A-kinase; lane 3, NF-L dephosphorylated by PP2A. Positions of molecular mass markers are indicated on the left of lane 1.

dephosphorylation suggests that NF-associated PP2A plays a role in preserving the filamentous form of NF in neurons.

Targeting of PP2A to NFs. The broad substrate specificity of protein phosphatases compared with protein kinases is one reason why their regulatory functions have not been investigated so extensively as those of protein kinases. However, it is gradually becoming apparent that the substrate specificities of protein phosphatases are more restricted than was previously thought. There seem to be two mechanisms by which protein phosphatases identify their specific substrates among cellular proteins: (1) targeting by close approximation of protein phosphatases to substrate proteins and (2) the highly restricted substrate specificity. Although the substrate specificities of protein phosphatases have been shown to be altered by bound regulatory subunits (Sola et al., 1991; Agostinis et al., 1992; Ferrigno et al., 1993), the association of PP2A with NFs that we show here would be an example of targeting. This association would facilitate the dephosphorylation of NF proteins in neuronal cytoskeletons, in which it would otherwise not be easy for PP2A to approach NFs.

This is the first clear evidence, to our knowledge, of association of PP2A with specific subcellular structures, although a PP2A-like enzyme (PP4) has recently been shown to be localized in centrosomes (Brewis et al., 1993). The association of PP1 with glycogen granules (PP1G) is well known (Stralfors et al., 1985; Tang et al., 1991). In that case, the regulatory subunit protein (called G) determines the binding of PP1 to glycogen granules (Hubbard & Cohen, 1989). Muscle PP1 has been shown to be a complex with a 130-kDa regulatory subunit that targets PP1 to myosin filaments (Alessi et al., 1992; Dent et al., 1992). In our case too, we thought that there should be a regulatory subunit that targets PP2A to NF, but we have not yet identified such a subunit protein. The NF-phosphatase released from NFs by 0.6 M $MgSO_4$ contained the 65-kDa protein as a regulatory subunit. Although the 65-kDa regulatory subunit can bind to proteins such as the B regulatory subunit or SV40 small t antigen (Yang et al., 1991), the NF-phosphatase might have another regulatory subunit, one of B regulatory subunits, beside the 65-kDa A subunit (Mayer-Jaekel & Hemmings, 1994) that determines the targeting of PP2A to NF because the complex of the catalytic and 65-kDa regulatory subunit prepared from the NF fraction did not rebind to either native NFs or reassembled NFs in a reconstruction experiment (data not shown).

It is now clear that formation of a specific complex of the PP2A catalytic subunit with A and B regulatory subunits plays an important role in regulating activity and substrate specificity. Reconstitution studies on cardiac PP2A have shown that the A and B subunits modulate activity toward a number of substrates (Imaoka et al., 1983; Chen et al., 1989). PP2A containing the B regulatory subunit ($PP2A_1$) shows higher activity toward histone H1 phosphorylated by cdc2 kinase than the catalytic subunit of PP2A (Sola et al., 1991; Agostinis et al., 1992; Ferrigno et al., 1993). We suspected that NF-associated PP2A might have higher activity on NF proteins than on other substrate proteins like phosphorylase *a*. However, the NF-associated PP2A showed similar relative activities in dephosphorylation of NF proteins and phosphorylase *a* to those of the purified catalytic subunit of PP2A (Table 1). It may be worth noting here that the NF-associated or purified catalytic subunit of PP2A did not dephosphorylate the phosphates in the tail domain of NF-H (data not shown). The tail domain of NF-H is phosphorylated by cdk5, a brain cdc2-like kinase (Lew et al., 1992; Hisanaga et al., 1993; Shetty et al., 1993). cdk5 has a similar substrate specificity to cdc2 kinase (Hisanaga et al., 1995). Since histone H1 phosphorylated by cdc2 kinase is dephosphorylated by PP2A complexed with the B regulatory subunit as described above (Sola et al., 1991; Agostinis et al., 1992; Ferrigno et al., 1993), a protein phosphatase for phosphorylation sites at the NF-H tail domain may bind a B regulatory subunit similar to that for cdc2 kinase-phosphorylated histone H1. Considering that an increasing number of B regulatory subunits have been reported (Mayer-Jaekel & Hemmings, 1994), PP2A with different B regulatory subunits would serve the head and the tail domain of NF proteins, respectively, for substrates.

Functional Role of the NF-Associated PP2A. The assembly of NF-L is regulated by phosphorylation of the head domain *in vitro*. Phosphorylation with A-kinase and C-kinase disassembles filaments reconstructed from NF-L into an oligomeric form, probably the octamer, and inhibits assembly of oligomeric forms of NF-L into filaments (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990).

Native NFs prepared from bovine spinal cord by gel filtration were fragmented by phosphorylation of the head domain of NF proteins by A-kinase (Hisanaga et al., 1994). However, the mechanism regulating NF-assembly/disassembly *in vivo* is not yet known. In this paper, we showed that native NFs prepared from bovine spinal cord associated with PP2A that removed phosphates introduced by A-kinase, and we suggest the role of PP2A in preserving NFs in a filamentous state.

With regard to the physiological meaning of NF-associated PP2A, at least three issues should be discussed. One is whether A-kinase is the kinase that phosphorylates the head domain of NF proteins *in vivo*. We used A-kinase-phosphorylated NF proteins in this experiment as a substrate for the NF-associated PP2A. C-kinase as well as A-kinase can inhibit the assembly of NF-L by phosphorylation *in vitro* (Gonda et al., 1990), but we have no information about the *in vivo* phosphorylation of NF proteins with C-kinase. cdc2 kinase has been suggested to be responsible for the phosphorylation of the N-terminal head domain of vimentin and keratin filaments at mitosis (Skalli et al., 1992). A cdc2-like kinase, known as cdk5, is also present in the brain (Lew et al., 1992; Shetty et al., 1993), but the head domains of NF proteins are poor substrates for cdc2 kinase and brain cdc2-like kinase/cdk5 (Hisanaga et al., 1991, 1993a). Phosphorylation of NF proteins with cdc2 kinase did not induce disassembly (Sihag & Nixon, 1990, 1991; Hisanaga et al., 1991). In contrast, most phosphorylation sites by A-kinase are located in the head domain of NF proteins (Hisanaga et al., 1994). One of the major phosphorylation sites, Ser55, was shown to be phosphorylated in rat optic nerve axons (Sihag & Nixon, 1991). Further, A-kinase is reported to be associated with NFs (Dosemeci & Pant, 1992). Thus from all these findings, the most probable kinase for phosphorylation of the head domain of NF proteins *in vivo* is A-kinase. If so, the use of A-kinase in the phosphorylation of NF proteins can be rationalized.

The second issue is whether there is any evidence for the involvement of protein phosphatase in the regulation of NF assembly *in vivo*. Phosphate incorporated into Ser55 has a relatively rapid turnover soon after its formation (Sihag & Nixon, 1991), suggesting the occurrence of dephosphorylation events at least at the Ser55 site of NF-L. In addition, when rat dorsal root ganglion neurons were treated with 0.1 μM okadaic acid, NF proteins became phosphorylated and solubilized, resulting in the disruption of NF networks in axons (Sacher et al., 1992, 1994). We also observed the disruption of NFs in rat cerebrum primary cultured neurons treated with 0.1 μM okadaic acid (unpublished observations), at the same concentration that NF-associated PP2A, but not purified PP1, was inhibited *in vitro* (Figure 5). However, the head domains of most NF subunit molecules in neurons are thought to be in an unphosphorylated state because NF proteins are rarely detected in soluble cytoplasmic fractions (Morris & Lasek, 1982; Nixon et al., 1989). Thus, it seems likely that protein phosphatase(s) preserve(s) the filamentous state of NFs in neurons by competing with A-kinase.

The third point to be considered is when and what roles phosphorylation-dependent disassembly of NF play in neurons. In contrast to other intermediate filaments, such as vimentin, whose phosphorylation is required for reorganization of vimentin filaments at mitosis (Skalli et al., 1992), the roles of phosphorylation of NF are unknown because of the apparent absence of overall and drastic reorganization of NF networks in neurons. Nixon and Shea (1992) suggested a role of phosphorylation in the process of incorporation of newly synthesized NF proteins into a NF network in axons from the finding that phosphates incorporated into the head domain of NF-L *in vivo* display relatively rapid turnover soon after its synthesis. If this is the case, NF proteins must be dephosphorylated when they are incorporated into the axonal cytoskeleton. PP2A might bind to and dephosphorylate NF proteins at the time of their incorporation into the axonal cytoskeleton. Another possible role of phosphorylation/dephosphorylation is in axonal transport of NF proteins. There are reported to be two populations of NFs in the axons, stationary and moving (Nixon & Logvinenko, 1986). The moving population of NFs is thought to consist of relatively small polymers of NF proteins. Microinjection of fluorescently labeled NF-L revealed that NF-L was transported as small oligomeric forms along axons (Okabe et al., 1993). Considering that NF proteins have a tendency to be incorporated into filamentous forms, transported NF oligomeric proteins should be phosphorylated in their head domain. NF-associated PP2A might dephosphorylate moving NF proteins when they are reincorporated into the axonal NF network.

ACKNOWLEDGMENT

We thank Dr. Mitsuo Ikebe for providing the catalytic subunit of protein phosphatase 1 and protein phosphatase 2A and Drs. M. Inagaki (Tokyo Metropolitan Institute of Gerontology), K. Tachibana, and K. Ohsumi for valuable suggestions and discussion.

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BI942630H