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Phosphorylation of Native and Reassembled Neurofilaments Composed of NF-L, NF-M, and NF-H by the Catalytic Subunit of cAMP-dependent Protein Kinase

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Phosphorylation of neurofilament-L protein (NF-L) by the catalytic subunit of cAMP-dependent protein kinase (A-kinase) inhibits the reassembly of NF-L and disassembles filamentous NF-L. The effects of phosphorylation by A-kinase on native neurofilaments (NF) composed of three distinct subunits: NF-L, NF-M, and NF-H, however, have not yet been described. In this paper, we examined the effects of phosphorylation of NF proteins by A-kinase on both native and reassembled filaments containing all three NF subunits. In the native NF, A-kinase phosphorylated each NF subunit with stoichiometries of 4 mol/mol for NF-L, 6 mol/mol for NF-M, and 4 mol/mol for NF-H. The extent of NF-L phosphorylation in the native NF was nearly the same as that of purified NF-L. However, phosphorylation did not cause the native NFs to disassemble into oligomers, as was the case for purified NF-L. Instead, partial fragmentation was detected in sedimentation experiments and by electron microscopic observations. This is probably not due to the presence of the three NF subunits in NF or to differences in phosphorylation sites because reassembled NF containing all three NF subunits were disassembled into oligomeric forms by phosphorylation with A-kinase and the phosphorylation by A-kinase occurred at the head domain of NF-L whether NF were native or reassembled. Disassembling intermediates of reassembled NF containing all three NF subunits were somewhat different from disassembling intermediates of NF-L. Thinning and loosening of filaments was frequently observed preceding complete disassembly. From the fact that the thinning was also observed in the native filaments phosphorylated by A-kinase, it is reasonable to propose the native NF is fragmented through a process of thinning that is stimulated by phosphorylation in the head domain of the NF subunits.

INTRODUCTION

Neurofilaments (NF) comprise a class of intermediate filaments (IF) that are expressed in neuronal cells (Steinert and Roop, 1988). NF are constructed from three kinds of subunit proteins (NF-H, NF-M, and NF-L of apparent molecular mass 70, 150, and 200 kDa, respectively, on sodium dodecyl sulfate-polyacrilamide gel electrophoresis [SDS-PAGE]). Similar to other IF proteins, each NF subunit is composed of three domains: an amino-terminal head, an α-helix rich central rod,

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[1] Abbreviations used: NF, neurofilament(s); IF, intermediate filament; A-kinase, the catalytic subunit of cAMP-dependent protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

and a carboxy-terminal tail. Although it has been shown that purified NF-L can polymerize into long intermediate-sized filaments in vitro (Geisler and Weber, 1981; Liem and Hutchison, 1982; Zackroff *et al.*, 1982; Minami *et al.*, 1984; Balin and Lee, 1991), recent transfection experiments indicate that NF-L is incapable of self-assembly in the absence of other neurofilament proteins in vivo (Ching and Liem, 1993; Lee *et al.*, 1993). The mechanism of NF-assembly therefore remains to be elucidated.

It has been shown in vitro that phosphorylation of NF-L by the catalytic subunit of cAMP-dependent protein kinase (A-kinase) and protein kinase C inhibited its polymerization into long filaments and also depolymerized filaments (Gonda *et al.*, 1990; Hisanaga *et al.*, 1990a; Nakamura *et al.*, 1990). The major A-kinase phosphorylation site has been identified as Ser-55 in the amino-terminal head domain of NF-L (Sihag and Nixon, 1991). These studies indicate that the assembly of NF is regulated by phosphorylation in the head domain, as has been demonstrated for other IF (Inagaki *et al.*, 1987, 1988; Evans, 1988; Geisler and Weber, 1988; for a recent review see Skalli *et al.*, 1992).

Neurofilaments, however, have the following three characteristics that are different from other IF. 1) NF are composed of three distinct subunits: NF-L, NF-M, and NF-H. The effect of phosphorylation by A-kinase on NF composed of these three proteins has not yet been studied. 2) The NF-M and NF-H subunits have sites in the tail domain that are phosphorylated by the second-messenger–independent kinases such as brain cdc2-like kinase/cdk5 and mitogen-activated protein (MAP) kinase (Hisanaga *et al.*, 1991, 1993; Roder and Ingram, 1991, 1993; Lew *et al.*, 1992; Miyasaka *et al.*, 1993; Shetty *et al.*, 1993). These tail domains have been suggested to be the sites that interact with neighboring NF and other cytoplasmic organelles (Hirokawa, 1982; Hirokawa *et al.*, 1984). These interactions may therefore be regulated by phosphorylation of the tail domain (Minami and Sakai, 1985; Eyer and Leterrier, 1988; Hisanaga and Hirokawa, 1990b; Hisanaga *et al.*, 1991). It would be interesting to determine whether the phosphorylation states of the tail domains affect the phosphorylation reaction at the head domain. 3) In contrast to cells in which phosphorylation of the head domain of IF proteins has been suggested to cause reorganization of IF network at mitosis (Chou *et al.*, 1989; Evans, 1989), NF are found in neurons, which are postmitotic cells (Nixon and Shea, 1992). Whether phosphorylation of the head domain of NF proteins has physiological significance is an important question that remains to be answered.

To address these questions, we investigated the effect of phosphorylation on native NF composed of NF-L, NF-M, and NF-H subunits. Unexpectedly, we found that most of the native NF did not disassemble into oligomeric forms following treatment with A-kinase in-spite of the fact that nearly the same extent of phosphate incorporation was observed as with NF-L alone. Instead, native NF showed indications of some fragmentation following phosphorylation. By contrast, reassembled NF containing NF-L, NF-M, and NF-H subunits were shown to undergo phosphorylation-dependent disassembly. We also report here structures of phosphorylation-induced disassembling intermediates of the reassembled NF containing each of the NF subunits.

MATERIALS AND METHODS

Preparation of native and reassembled NF

Native NF was prepared as described previously (Hisanaga and Hirokawa, 1988). Bovine spinal cords were homogenized in equal volumes of PEM (0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) [PIPES], pH 6.8, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA]) containing 0.5 mM dithiothreitol (DTT), 1 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml leupeptin). A crude extract was obtained by centrifugation at 28 000 × *g* for 50 min at 4°C. The supernatant was separated by gel-filtration chromatography on Sepharose CL-4B directly in 10 mM PIPES, 1 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM DTT, 0.1 mM PMSF, and 1 μg/ml leupeptin or after dialyzing against dephosphorylation buffer (0.15 M NaCl, 50 mM tris(hydroxymethyl(aminomethane [Tris]-HCl, pH 8.5, 1 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM DTT, 0.1 mM PMSF, and 1 μg/ml leupeptin). Flow-through fractions were used as the source of native NF.

Dephosphorylation was performed as described previously (Hisanaga and Hirokawa, 1989). After dialysis against the dephosphorylation buffer, the crude extract was incubated with *Escherichia coli* alkaline phosphatase (Wako, Osaka, Japan) for 5 h at 35°C. At the end of the incubation, the reaction mixture was gel-filtered on Sepharose CL-4B and the flow-through fractions were used as the source of dephosphorylated, native NF.

Reassembled NF were prepared as follows. The crude extract was warmed to 35°C for 30 min and NF were collected by centrifugation at 150 000 × *g* for 60 min at 4°C. Pelleted NFs were solubilized in 8 M urea and 10 mM K-phosphate buffer (pH 7.5). After a brief centrifugation at 48 000 × *g* for 30 min, the supernatant fraction was stored at −80°C until use. Aliquots of the solubilized NF proteins were polymerized by dialyzing against reassembly solution (0.15 M NaCl, 10 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM DTT, 0.1 mM PMSF, and 1 μg/ml leupeptin) for 3 h at 35°C and gel-filtered on Sepharose CL-4B. The flow through fractions were used as the source of reassembled NF.

Phosphorylation of NF

NF proteins (~0.25 mg/ml) were incubated with the catalytic subunit of cAMP-dependent protein kinase (28–40 μg/ml) in 0.1 mM ATP, 1 mM $MgCl_2$, and 20 mM PIPES (pH 7.0) at 25°C (Inagaki *et al.*, 1988; Hisanaga *et al.*, 1990a). The reaction was stopped by adding SDS-PAGE sample buffer and the proteins resolved by electrophoresis in a 7.5% SDS-polyacrylamide gel. For measurements of radioactivity incorporated into NF proteins, NF proteins were excised from gels after staining with Coomassie Brilliant Blue and counted by Cerenkov radiation. For autoradiography, the dried gels were exposed to X-ray film at −80°C.

Chymotrypsin Digestion of Native NF

Phosphorylated NF were digested with α-chymotrypsin at a protein ratio of 625:1 at 30°C for 15 min as described previously (Hisanaga *et al.*, 1991). The reaction was stopped by the addition of PMSF to 5

mM. The tail domains released were separated from core filaments by centrifugation at 45 000 × *g* for 20 min.

One-dimensional Phosphopeptide, Amino Acid Sequence, and Amino Acid Composition Analysis of NF-L-derived Phosphopeptides

Porcine NF-L (0.15 mg/ml) phosphorylated by A-kinase was digested at 30°C overnight with lysil endopeptidase (2.5 μg/ml or 1.6 μg/mg NF-L). An aliquot was separated on 20% SDS-polyacrylamide gel for one-dimensional phosphopeptide analysis.

Fragmented NF-L was applied to a ODS-AP reverse-phase high performance liquid chromatography (HPLC) column (Zorbax C8, YMC, Kyoto, Japan) and was eluted with a linear gradient of 0–50% acetonitrile. Two radioactive peaks appeared. One of them was directly sequenced and its amino acid composition was analyzed as described by Ando *et al.* (1989). Another broad radioactive peak was combined and further digested by trypsin. Radioactive fragments were purified by a reverse-phase HPLC column and a DEAE-5WP anion-exchange column (Toso, Tokyo, Japan). The resultant two radioactive peaks were sequenced, and their amino acid compositions were analyzed (Ando *et al.*, 1989).

Phosphorylated NF-L was excised from 7.5% SDS-polyacrylamide gel in which phosphorylated native or reassembled NF proteins had been resolved. After washing with 25% isopropanol and 10% methanol, the gel containing NF-L was dried at 64°C and digested by lysil endopeptidase at a protein ratio of 100:1 in 50 mM Tris-HCl, pH 9.3, and 6 M urea. Digestion products were separated on 20% SDS-polyacrylamide gel and autoradiographed.

Electron Microscopy

Low-angle rotary shadowing was performed by the method of Tyler and Branton (1980) as described previously (Hirokawa, 1986; Hisanaga and Hirokawa, 1988). At the end of the phosphorylation reaction, 10 mM EDTA was added to stop the reaction. Phosphorylated NFs were diluted to a protein concentration of ~75 μg/ml with 50% glycerol. Samples (50–75 μl) were sprayed onto a newly cleaved mica surface and rotary shadowed with platinum (2.5 nm) at an angle of 6° in a Balzers BAF 301. Replicas were examined with a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan).

SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (1970). The amounts of NF triplet proteins were estimated by densitometric scanning of gels stained by Coomassie Brilliant Blue with a Dual Wavelength Flying Spot Scanner CS-9000 (Shimazu, Osaka, Japan).

RESULTS

Phosphorylation of the Native NF

Native NF were prepared from crude extracts of bovine spinal cords as flow-through fractions from a Sepharose CL-4B gel-filtration column (Hisanaga and Hirokawa, 1988). When incubated with the catalytic subunit of A-kinase, each of NF subunit proteins was shown to be phosphorylated. Phosphate incorporation into NF subunit proteins reached a maximum at ~10–20 min and then declined gradually when examined up to 120 min (Figure 1). The decline in phosphorylation levels suggests that the native NF fraction contained a phosphatase that removed phosphates from A-kinase phosphorylation sites.

The effect of phosphorylation on the filamentous structures was studied by means of negative staining and rotary shadowing. Disassembly of the filament into oligomeric forms was not observed after phosphorylation, either at the time of maximum phosphorylation or after 120 min incubation.

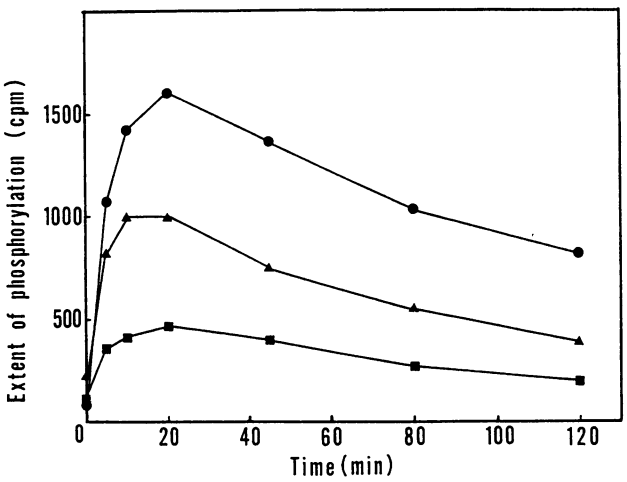

Figure 1. Time course of phosphorylation of native NF by A-kinase. Native NF (0.25 mg/ml) was incubated with A-kinase (19 μg/ml) at 35°C. The reaction was stopped by addition of SDS-PAGE sample buffer at the indicated times and the samples were analyzed by 7.5% SDS-PAGE. Radioactivity incorporated into each NF protein was determined by Cerenkov radiation counting of the excised bands (●, NF-L; ▲, NF-M; ■, NF-H).

Because there is a possibility that no disassembly of the filament following phosphorylation could be due to a dephosphorylation reaction occurring concomitantly with the phosphorylation reaction, we attempted to conduct this experiment in the absence of phosphatase activity. We found that the phosphatase activity in the native NF fraction disappeared when the spinal cord extract was dialyzed against the dephosphorylation buffer at pH 8.5 before gel-filtration. When we repeated the phosphorylation experiments with native NF dialyzed against the dephosphorylation buffer, the phosphorylation pattern was generally similar to that shown in Figure 1 except for the continuance of the plateau level for 120 min (Figure 2B). Glial fibrillary acidic protein (GFAP) with a molecular mass of ~52K, which contaminated the NF fraction, was also phosphorylated to some extent. Total phosphate incorporations were in the order of NF-L, NF-M, and NF-H (Figure 2C). In the absence of A-kinase, only a background level of phosphorylation was detected for each triplet protein under our incubation conditions.

The extent of phosphorylation per mol subunit of protein was calculated by measuring the amounts of protein in the gel by a densitometer, assuming the molecular masses of NF-L, NF-M, and NF-H to be 62K, 95.6K, and 110K, respectively (Figure 2D) (Geisler *et al.*, 1985; Napolitano *et al.*, 1987; Lees *et al.*, 1988). Interestingly, the specific radioactivities of each subunit fell within a very narrow range (4 mol P/mol NF-L, 6

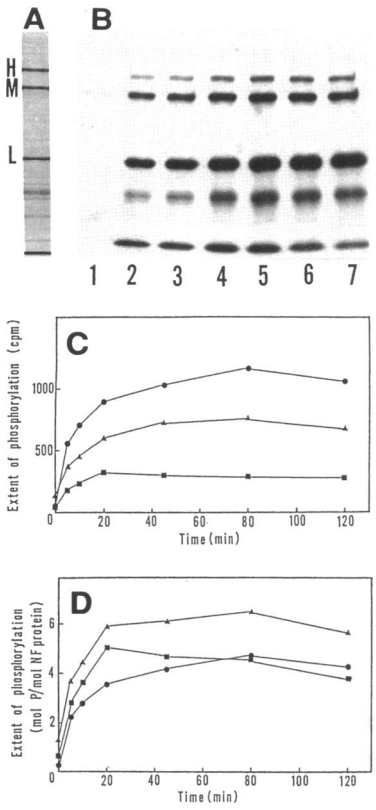

Figure 2. Phosphorylation by A-kinase of native NF, dialyzed against alkaline pH to eliminate endogenous phosphatase activity. (A) Protein staining of the NF fraction. NF subunits, NF-L, NF-M, and NF-H, are designated L, M, and H, respectively. (B) Autoradiograph of the phosphorylated NF proteins. Phosphorylation was performed as described in Figure 1 at 35°C for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 45 min (lane 5), 80 min (lane 6), and 120 min (lane 7). (C) Radioactivity incorporated into each NF protein. Cerenkov radiation in excised gels was counted by a liquid scintillation counter (●, NF-L; ▲, NF-M; ■, NF-H). (D) The extents of phosphorylation per mole protein. The extents of phosphorylation per mole of subunit protein was estimated from the total radioactivity shown in D assuming molecular masses of NF-L, NF-M, and NF-H as 62 000, 95 600, and 110 000, respectively.

mol P/mol NF-M, and 4 mol P/mol NF-H). The number of phosphorylation sites in NF-L in the native NF was almost the same as that obtained with purified NF-L (Hisanaga *et al.*, 1990a).

Rapid incorporation of phosphates into NF-M was consistently observed. Even at 0 min incubation (we usually took ~20 s to stop the reaction by addition of SDS-PAGE sample buffer after starting the reaction by addition of [γ-^{32}P]ATP), a small but reproducible labeling was detected in NF-M (Figure 2B, lane 1, and Figure 2C, 0 min).

To test the effect of phosphorylation on filaments, a centrifugation experiment was carried out. Centrifugation at 90 000 × *g* for 60 min, which was used to determine the extent of disassembly of NF-L in a previous study (Hisanaga *et al.*, 1990a), precipitated almost all phosphorylated NF (Figure 3, lane 4), indicating that disassembly into oligomeric forms did not occur. When the centrifugation force was reduced to 53 000 × *g* for 30 min, only a little more phosphorylated NF proteins than unphosphorylated NF was detected in the supernatant (Figure 3, lane 7). This suggested that the number of shorter NF increased after phosphorylation.

Effects of phosphorylation on filaments were examined by negative staining and rotary shadowing electron microscopy. Rotary shadowed images of the native NF before and after phosphorylation are shown in Figure 4. General filamentous structures (22-nm beaded segments along filaments and many lateral projections) were similar to those described previously (Figure 4a) (Hisanaga and Hirokawa, 1988). Phosphorylation did not disassemble the native NF into rods. The native NF remained as filaments even after they were phosphorylated (Figure 4b). Although the shorter filaments seemed to be more abundant after phosphorylation, this could not be quantified because fragmentation of filaments occurred upon spraying onto mica.

We sometimes observed thinner regions in the native NF after phosphorylation. A typical example is shown in Figure 5. These thinner regions might be sites where the native NF broke after phosphorylation (see below).

Phosphorylation of the Dephosphorylated, Native NF by A-kinase

It is of interest to determine whether the phosphorylation states of the carboxy-terminal tail domain of NF-M and NF-H affect the A-kinase-dependent phosphorylation of the head domain. The dephosphorylated NFs were prepared as described in a previous paper (Hisanaga and Hirokawa, 1989). Because dephosphorylation increases the mobility shift of NF-H to a much greater degree than that of NF-M, the dephosphorylated NF-M and NF-H proteins migrated more closely together on SDS-PAGE than that observed for the same subunit derived from native NF (cf. Figure 6A to Figure 2A).

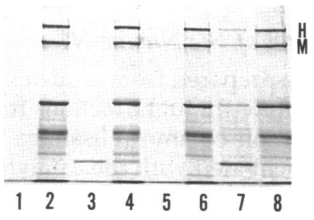

Figure 3. A sedimentation experiment with native NF phosphorylated by A-kinase. Phosphorylation of native NF pre-exposed to alkaline pH was performed at 35°C for 120 min as described in Figure 1. Phosphorylated (lanes 3, 4, 7, and 8) and unphosphorylated (but incubated in the absence of A-kinase) (lanes 1, 2, 5, and 6) NF were centrifuged at 90 000 × *g* for 60 min (lanes 1–4), or 53 000 × *g* for 30 min (lanes 5–8). The resulting supernatant fractions (lanes 1, 3, 5, and 7) and precipitates (lanes 2, 4, 6, and 8) were resolved by 7.5% SDS-PAGE. NF-L, NF-M, and NF-H are designated L, M, and H, respectively.

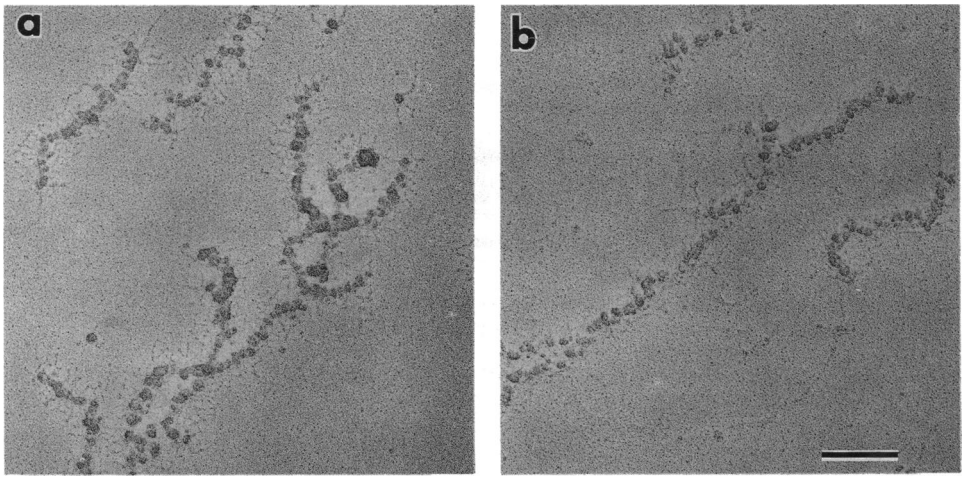

Figure 4. Rotary-shadowed electron micrographs of the untreated and the phosphorylated NF. Phosphorylation of the native NF in the absence of the phosphatase activity was performed as described in Figure 1 for 120 min. The untreated and the phosphorylated NF were processed for rotary shadowing as described previously (Hisanaga and Hirokawa, 1988). Many filaments were observed before phosphorylation (a) and after phosphorylation (b) by A-kinase for 120 min. The number of shorter filaments seemed to be increased after phosphorylation, however. Bar, 200 nm.

Under the reaction conditions used, phosphorylation appeared to be accomplished in 20 min and the plateau level was maintained for a further 100 min (Figure 6C). Despite exhaustive dephosphorylation of NF-M and NF-H, the phosphate incorporation pattern in the dephosphorylated NF fraction was very similar to that observed with the native NF (Figure 6B). As observed for native NF, total phosphate incorporation increased in the order of NF-L, NF-M, and NF-H. The number of phosphorylation sites per mole protein was 2–3 mol for each protein (Figure 6C). These were even smaller than the values obtained with the native NF, in spite of the increase of available phosphorylation sites in the tail domain. This result confirms that A-kinase does not recognize the major phosphorylation sites in the tail domain of NF-M and NF-H as a substrate (Hisanaga *et al.*, 1991).

Electron microscopic observations revealed that phosphorylation of the dephosphorylated, native NFs by A-kinase did not disassemble filaments into oligomeric forms.

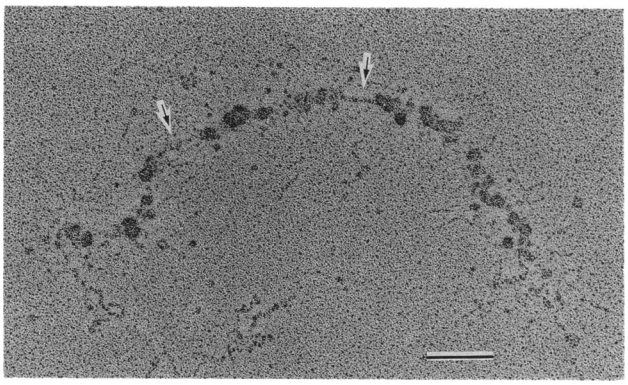

Figure 5. A high-magnification view of native NF phosphorylated by A-kinase to show the thinning in the middle of the filaments. Phosphorylation of native NF was performed for 120 min as described in Figure 1. The thinning regions are indicated by arrows. Rotary shadowing was carried out as described in MATERIALS AND METHODS. Bar, 100 nm.

Phosphorylation of the Reassembled NF

In contrast to the filaments reassembled from NF-L, the native filaments did not disassemble into oligomeric forms after phosphorylation with A-kinase. To examine the differences in sensitivity to phosphorylation-me-

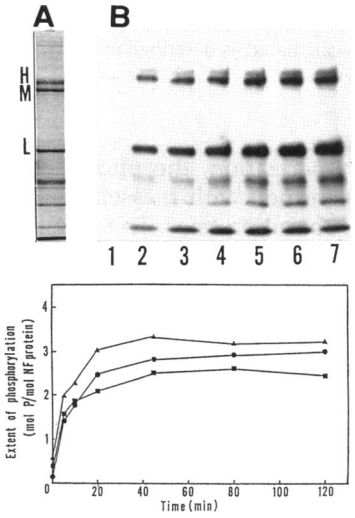

Figure 6. Phosphorylation of dephosphorylated, native NF by A-kinase. (A) Protein staining of the dephosphorylated, native NF fraction. Dephosphorylated NF-L, NF-M, and NF-H are designated L, M, and H, respectively. (B) Autoradiograph of the dephosphorylated form of NF phosphorylated by A-kinase. Phosphorylation was performed as described in Figure 1 for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 45 min (lane 5), 80 min (lane 6), and 120 min (lane 7). (C) Radioactivity incorporated into each dephosphorylated NF protein. Cerenkov radiation in excised gel slices was counted using a scintillation counter. The extent of phosphorylation per mole protein was calculated as described in Figure 2. ●, NF-L; ▲, NF-M; ■, NF-H.

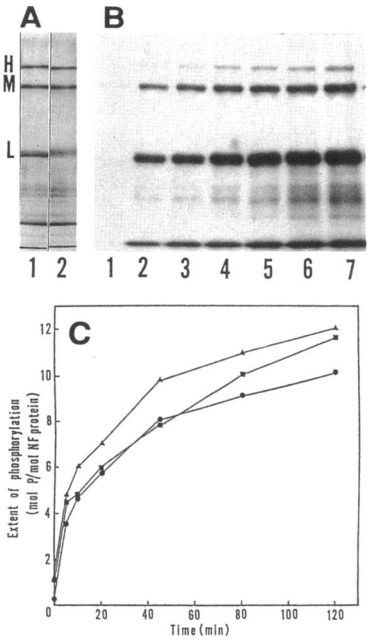

Figure 7. Phosphorylation of the reassembled NF by A-kinase. (A) Protein staining of the reassembled NF before (lane 1) and after (lane 2) phosphorylation. NF-L, NF-M, and NF-H are designated L, M, and H, respectively. (B) Autoradiograph of the reassembled NF proteins phosphorylated by A-kinase. The reassembled NF (0.25 mg/ml) was incubated with the catalytic subunit of A-kinase (40 μg/ml) at 35°C for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 45 min (lane 5), 80 min (lane 6), and 120 min (lane 7). (C) Radioactivity incorporated into each NF protein. Cerenkov radiation in excised gel slices was counted using a scintillation counter. The extent of phosphorylation was calculated as described in Figure 2. ●, NF-L; ▲, NF-M; ■, NF-H.

diated depolymerization, the phosphorylation experiment was performed with reassembled NF containing all of the NF subunit proteins. The reassembled NF were prepared by one cycle of urea-dependent disassembly-reassembly from the crude extract followed by gel filtration on Sepharose CL-4B without further purification of the subunit proteins.

We initially carried out phosphorylation reactions using ratios of enzyme to substrate similar to that used with the native NF. However, incorporation of radioactivity into the subunit proteins did not reach a plateau even after incubation for 120 min. Therefore, the amount of enzyme used for the phosphorylation was increased. The result presented in Figure 7 was obtained in experiments in which the amount of A-kinase was increased twofold. Phosphorylation proceeded rapidly during the first 5–10 min, after which the rate of phosphorylation decreased gradually, but the extent of phosphorylation was still increasing after 120 min (Figure 7C). About 4–6 mol of phosphate per mole protein was rapidly incorporated into NF proteins (Figure 7C), an amount that corresponds to the extent of phosphorylation obtained with the native NF. After 120 min incubation, the amount of phosphorylation increased to 10–12 mol/mol protein (Figure 7C), ~2.5-fold that of native NF.

We noticed here that NF-L changed its electrophoretic mobility after phosphorylation. Protein staining of the NF proteins before and after phosphorylation is shown in Figure 7A. Although NF-M and NF-H did not change their electrophoretic mobility, the phosphorylated NF-L formed diffuse bands at a little higher molecular weight region. Furthermore, the shifted bands contained more phosphates than those in the lower molecular weight region.

The effect of phosphorylation on the filaments was studied by a sedimentation assay at 100 000 × *g* for 1 h. In contrast to the native NF, NF proteins remaining in the supernatant increased with phosphorylation (lanes 1, 3, 5, 7, and 9 in Figure 8, A and B), although more than one-half the NF were still precipitated after 120 min incubation (Figure 8A, lanes 9 and 10). Two interesting aspects were revealed by comparing NF proteins in the supernatant fraction (lane 9) to those in the precipitate (lane 10): 1) NF-L in the supernatant fraction showed a slower electrophoretic mobility than that in the precipitates, suggesting that highly phosphorylated NF-L has a tendency to disassemble. 2) NF-M and NF-H partitioned differently into the supernatant fraction and the precipitate by centrifugation after phosphorylation. Although more NF-M remained in the supernatant fraction than in the precipitate, more NF-H appeared in the precipitate than in the supernatant fraction (Figure 8, lanes 9 and 10).

Because the sedimentation assay had a tendency to underestimate the extent of disassembly compared with the electron microscopic observation, as we have indicated in a previous paper (Hisanaga *et al.*, 1990a), disassembly of the reassembled NF by phosphorylation was further examined by negative staining and rotary

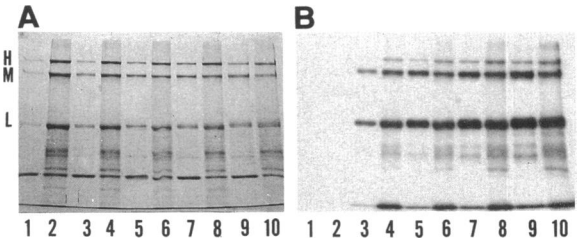

Figure 8. SDS-PAGE analysis of the disassembly of the reassembled NF by phosphorylation. Phosphorylation of the reassembled NF was performed as described in Figure 7 for 0 min (lanes 1 and 2), 5 min (lanes 3 and 4), 20 min (lanes 5 and 6), 45 min (lanes 7 and 8), and 120 min (lanes 9 and 10). The reaction was stopped by adding EDTA at a final concentration of 5 mM. After centrifugation at 90 000 × *g* for 60 min, the supernatant fractions (lanes 1, 3, 5, 7, and 9) and the precipitates (lanes 2, 4, 6, 8, and 10) were resolved by 7.5% SDS-PAGE. Protein staining and an autoradiograph are shown in A and B, respectively. NF-L, NF-M, and NF-H are indicated in A by L, M, and H, respectively.

shadowing electron microscopy. Only the micrographs from rotary shadowing experiments are shown in Figure 9. The reassembled NF contained intermediate-sized filaments with extended lateral projections as a major component and a small amount of globular particles (Figure 9a). One or both ends of filaments frequently became thicker or terminated with globular structures. The reassembled NF incubated for 5 min with A-kinase are shown in Figure 9b. The number of short filaments increased. Incubation with A-kinase for 20 min depolymerized the filaments and only the small globular structures were identified. Rod-like oligomeric structures became predominant after further incubation up to 120 min (Figure 9d). These oligomeric structures might correspond to the eight-chain complex (Hisanaga *et al.*, 1990a,b), although in the case of other intermediate filaments the basic building block is indicated to be the four-chain complex (Steinert and Roop, 1988).

Higher Magnification Views of Disassembling Filaments

We reported that filamentous NF-L depolymerizes into a eight-chain complex through unraveled structures as intermediates by phosphorylation with A-kinase (Hisanaga *et al.*, 1990a). However, the filaments composed of triplet proteins appeared to take somewhat different disassembly pathways from the filaments reconstructed from NF-L alone. We did not observe the star-like clusters and the distinct unraveled filaments shown with filamentous NF-L (see Figure 5 in Hisanaga and Hirokawa [1990a]). In places, we recognized two different kinds of disassembling intermediates as shown in Figure 10. One was the thinning of the filaments (Figure 10, a and b), and another the loosing of the filaments (Figure 10, c and d). In the thinning regions, subfilaments appeared to be peeled from the core filament. The loosing regions also appeared in the middle and at the ends of

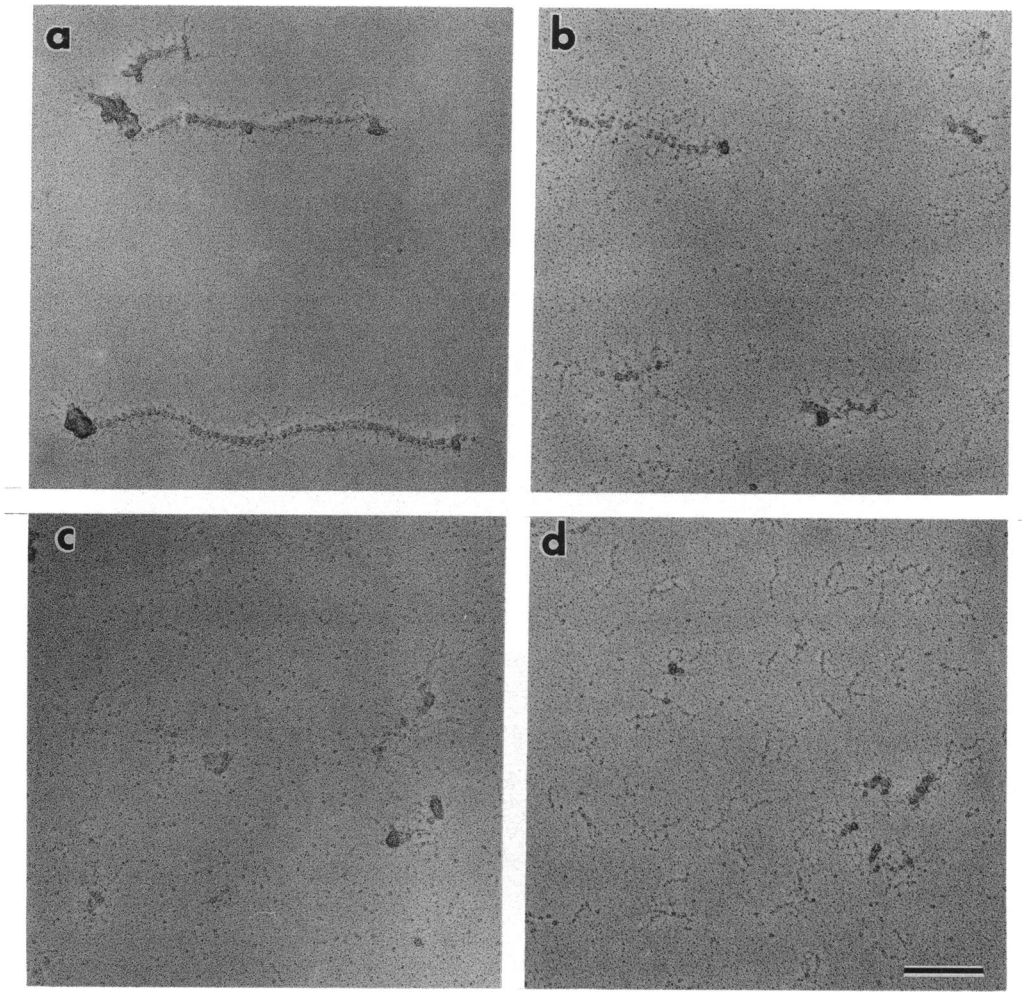

Figure 9. Rotary-shadowed electron micrographs of the reassembled NF phosphorylated by A-kinase. Phosphorylation was performed as described in Figure 7 for 0 min (a), 5 min (b), 20 min (c), and 120 min (d). The reassembled NF were processed for rotary shadowing at indicated times of phosphorylation reaction as described in MATERIALS AND METHODS. Bar, 200 nm.

the filaments, where distinct 22-nm repeats were not identified clearly. The disassembling intermediate of the thinning was very similar to that observed with phosphorylated, native NF as described above (Figure 5).

A-Kinase-phosphorylated Domains of NF Proteins

One of the particular characteristics of NF proteins is the presence of very long carboxy-terminal tail domains of the higher molecular weight mass subunits, NF-H and NF-M. First, we examined in more detail whether the carboxy-terminal tail domain of NF-H and NF-M are not phosphorylated by A-kinase. The carboxy-terminal tail domains that extend outward from core filaments were separated from core filaments by a brief digestion with α-chymotrypsin. Chymotryptic digestion of phosphorylated native NF produced new bands (Figure 11A, lane 2), which are indicated by h and m in lane 4. When the chymotrypsin-treated NF were centrifuged to sediment the remaining filaments, the released tail domains of h and m remained in the supernatant (Figure 11A, lane 4). Autoradiography (Figure 11A, lanes 5–8) indicated that some phosphorylation occurred in the tail domain of NF-M (m in lane 8), whereas no phosphorylation was detected with the NF-H tail domain (h in lane 8).

Phosphorylation sites of NF-L was determined using porcine NF-L because its amino acid sequence is already known. Porcine NF-L phosphorylated by A-kinase was digested by lysil endopeptidase and analyzed by 20% SDS-PAGE (Figure 11B). Lysil endopeptidase digests produced phosphopeptides with molecular masses of ~9500–13 500 (Figure 11B, lane 2). More than 95% of the ^{32}P incorporated into NF-L was recovered in these phosphopeptides. Close inspection of the amino acid sequence of NF-L (Geisler *et al.*, 1985) indicated that these long peptides were derived from the amino-terminal head domain, suggesting phosphorylation also occurs in the head domain.

To confirm this, phosphopeptides were separated by reverse-phase chromatography. Two radioactive peaks appeared and the fragment in the first peak was identified from its amino acid composition as residues 1–14 (Figure 12A). The second broad peak was further digested with trypsin and again separated by a reverse-phase column (Figure 12B). The first large peak was shown by direct sequencing to contain residues 37–53. The second large peak was further purified by chromatography in a DEAE-5WP column and was identified by sequencing to be residues 55–82. Because all these major phosphopeptides were derived from the head domain, we concluded that A-kinase predominant phosphorylation sites of NF-L are at the head domain.

We also examined whether or not different sites of native and reassembled NF are phosphorylated by A-kinase. Because the most abundant subunit among trip-

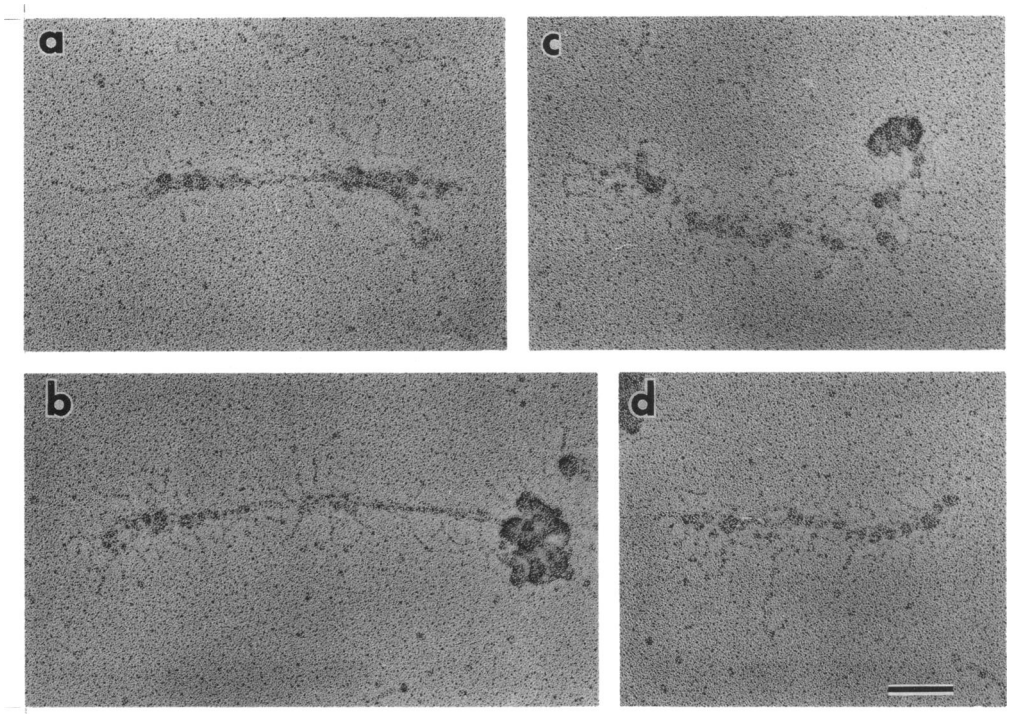

Figure 10. Higher magnification views of the reassembled NF in the process of phosphorylation-mediated disassembly. Phosphorylation was performed as described in Figure 7 for 5 min. Rotary shadowing was carried out as described in MATERIALS AND METHODS. Two kinds of disassembly intermediate were identified: thinning (a and b) and loosening (c and d). Bar, 100 nm.

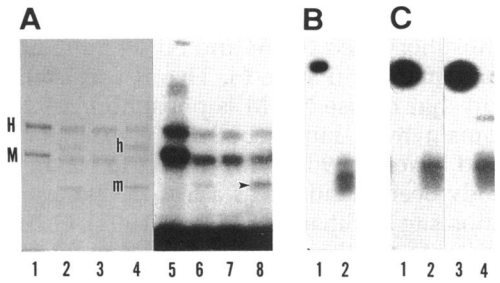

Figure 11. Identification of the phosphorylated domain in NF proteins by A-kinase. (A) Protein staining (lanes 1–4) and autoradiograph (lanes 5–8) of 5% SDS-PAGE of native NF phosphorylated by A-kinase and thereafter digested by α-chymotrypsin. Lanes 1 and 5: NF proteins phosphorylated by A-kinase; lanes 2 and 6: chymotrypsin-digested NF proteins; lanes 3 and 7: precipitation of chymotrypsin-digested NF after centrifugation at 45 000 × *g* for 20 min; lanes 4 and 8, the supernatant fractions containing the tail domains of NF-H and NF-M. NF-H, NF-M, and the tail domain of NF-H and NF-M are indicated by H, M, h, and m, respectively. Although no phosphorylation was detected in the NF-H tail domain, some phosphorylation occurred in the NF-M tail domain (lane 8, arrowhead). (B) One-dimensional phosphopeptide analysis of porcine NF-L phosphorylated by A-kinase. Porcine NF-L was phosphorylated at 25°C for 20 min by A-kinase (lane 1), digested at 30°C overnight by lysil endopeptidase (lane 2), and autoradiographed after 20% SDS-PAGE. Molecular weight markers: 97.4, 66.2, 42.7, 31.0, 21.5, and 14.4. (C) One-dimensional phosphopeptide analysis of A-kinase-phosphorylated NF-L from native NF and reassembled NF. Native (lanes 1 and 2) and reassembled (lanes 3 and 4) NF were phosphorylated by A-kinase and NF subunit proteins were resolved by 7.5% SDS-PAGE. Excised NF-L was digested as described in MATERIALS AND METHODS by lysil endopeptidase (lanes 2 and 4) and rerun on 20% SDS-PAGE together with undigested NF-L (lanes 1 and 3).

let proteins is NF-L and its phosphorylation sites were shown to be at the head domain as described above, the phosphorylation domain of NF-L was determined on a one-dimensional phosphopeptide map after lysil endopeptidase digestion (Figure 11C). A phosphopeptide pattern from the reassembled NF-L filaments was almost the same as that from the native NF, although a quantitative differences were noted (Figure 11C, lanes 2 and 4). This result suggests that the major A-kinase phosphorylation sites in NF-L are in the amino-terminal head domain in both the native and reassembled filaments.

DISCUSSION

In this paper, we reported that native NF were not disassembled into oligomeric forms after phosphorylation with A-kinase. This is in contrast to the results obtained with filaments reassembled from NF-L, which disassembled into the eight-chain complex after phosphorylation (Hisanaga *et al.*, 1990b). This difference is not due to the number of subunit proteins in the filaments or the phosphorylation domain. Reassembled NF containing all three subunit proteins were shown here to depolymerize into oligomeric structures after phosphorylation. NF-L was phosphorylated at the head domain in both native or reassembled NF.

There was a difference in the extent of phosphorylation between the native and reassembled NF in that 2.5–3 times as many phosphates were incorporated into NF proteins in the reassembled NF (Figure 7). This additional phosphorylation should occur at sites that are exposed only in the reassembled NF. We think at present that overphosphorylation is an in vitro artifact observed only with reassembled NF and could be one of indications suggesting differences in filamentous state of NF. It could be possible that phosphorylation at these additional sites induced the reassembled NFs to disassemble into oligomers. However, because the reassembled NF began to disassemble at 20 min incubation with A-kinase (Figure 9c) when only four to six phosphates were incorporated into each NF protein (before additional phosphorylation) (Figure 7C), we think there should be some distinct differences in the sensitivity to phosphorylation between native and reassembled NF. We think the urea treatment that changes the sensitivity in disassembly by phosphorylation. Differences in the stability of native and reassembled filaments against urea treatment has been reported for epidermal keratin by Eichner and Kahn (1990).

There could be two possible explanations for the differential sensitivity against phosphorylation that might be produced by the urea treatment. One is that there

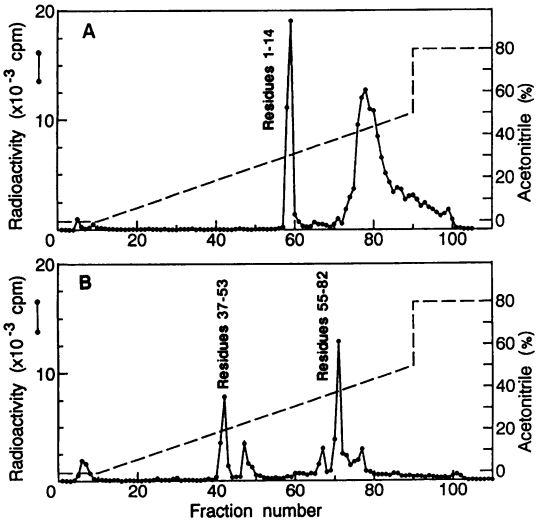

Figure 12. Reverse-phase HPLC profile of phosphopeptide fragments of NF-L. Porcine NF-L phosphorylated by A-kinase was digested with lysil endopeptidase, applied to a ODS-AP reverse-phase column, and eluted with a linear 0–50% acetonitrile gradient in 0.1% trifluoroacetic acid (A). The first peak was identified as residues 1–14 from its amino acid composition. The second broad peak was further digested with trypsin and separated by a ODS-AP reverse-phase column (B). The first large radioactive peak was directly sequenced and identified as residues 37–53. The second large radioactive peak was further purified using a DEAE-5WP column and identified as residues 55–82 by sequencing.

are structural differences between native and reassembled NF. Although there is no direct evidence for such differences, it is possible that the urea treatment alters the structure responsible for the phosphorylation-dependent disassembly process. Another possibility concerns the effect of NF-associated proteins. Our native NF preparation contained proteins other than the three NF subunits. Many of these proteins appeared to be associated with NF. When NF were pelleted by low-speed centrifugation (16 000 rpm, 20 min), almost all of these proteins coprecipitated together with NF. Some of these associated proteins, which also make the native NF resistant against phosphorylation-dependent disassembly, might be denatured by the urea treatment.

In a previous paper (Hisanaga and Hirokawa, 1990a), we presented a model for the reassembly of NF-L protein in vitro. Phosphorylation-induced disassembly of filamentous NF-L appeared to go through a process that is the reverse of assembly (Hisanaga *et al.*, 1990a). That is, we observed star-like clusters and unraveled filaments in the process of disassembly, that were initially identified as NF-L-assembly intermediates. In places, however, we observed thinning and loosing of the filaments in the intermediate structures with the reassembled filaments that contained all of the three NF proteins. Although we do not know the reason for the difference in the intermediate structures between filaments composed of NF-L alone and all three subunits, this might not simply be due to the addition of either NF-M or NF-H to NF-L because the filaments composed of NF-L plus either NF-M or NF-H unraveled when they were polymerized in the presence of 50 mM NaCl and in the absence of $MgCl_2$ (Hisanaga and Hirokawa, 1990a). NF-associated proteins might also affect the disassembling intermediate structures. We observed thinner regions in the phosphorylated, native filaments and the length of the native NFs seemed to become shorter after phosphorylation. Taken together, these data suggest that severing may occur at the thinner regions and that this type of the severing of NF may reflect an in vivo response to phosphorylation.

In addition to the two major findings discussed above, we found several interesting aspects of the phosphorylation reaction of NF proteins. First, the native NF fraction contained a phosphatase(s) that can remove phosphates from sites phosphorylated by A-kinase. The action of such a phosphatase(s) in neurons would move the NF assembly/disassembly equilibrium toward assembly through dephosphorylation of the head domain. If this is the case, it is reasonable to guess that the amount of the soluble forms of NF proteins are low in neurons.

Second, most of the A-kinase phosphorylation sites appear to be located in the head domain of NF proteins. Although some phosphorylation occurs in the NF-M tail domain, the major in vivo phosphorylation sites at the tail domain of NF-M and NF-H were not good substrates for A-kinase. A-kinase did not phosphorylate the dephosphorylated NF-M and NF-H more than the phosphorylated NF (Figure 6). This is consistent with the report that mouse NF-M is phosphorylated in the head domain by A-kinase both in vivo and in vitro (Sihag and Nixon, 1990). With respect to NF-L, Ser-55 has already been identified as a major A-kinase phosphorylation site by Sihag and Nixon (1991). Our results are consistent with their data and indicate further that other phosphorylation sites of NF-L in addition to Ser-55 also reside in the head domain.

Third, each triplet polypeptide showed a different response to phosphorylation. 1) NF-M appeared to be the best substrate for A-kinase. NF-M was phosphorylated to a larger extent and more rapidly than either NF-L or NF-H. Preferential phosphorylation of NF-M by microtubule-associated cAMP-dependent protein kinase has already been reported (Leterrier *et al.*, 1981). 2) NF-L in reassembled NF showed a decreased electrophoretic mobility upon phosphorylation with A-kinase, although this could be due to in vitro overphosphorylation observed only with reassembled NF. 3) Compared with NF-H, NF-M appeared to be more easily released from core filaments by phosphorylation. Differential solubilization of NF-M and NF-H by phosphorylation might reflect the inhomogeneity of three NF subunit proteins along the filaments, although we have not detected any regional differences in the density of projections in either the native or the reassembled NF (Hisanaga and Hirokawa, 1988). A soluble variant of NF-M (S150) has been isolated from bovine brain NF preparation by Wong *et al.* (1984). However, solubilization of NF-M after phosphorylation described here may be distinct from their observation, because S150 was inferred to be solubilized as a result of dephosphorylation.

Our observation that the native NF were fragmented, but not disassembled, by phosphorylation with A-kinase might reflect a reaction more close to the one occurring in vivo compared with the results obtained with reassembled NF. Considering that the A-kinase phosphorylation sites in vitro are phosphorylated in vivo in the mouse optic axon (Sihag and Nixon, 1990, 1991) and that A-kinase associates with isolated NF (Dosemeci and Pant, 1992), phosphorylation at the head domain by A-kinase could occur to some extent in vivo. What is the physiological meaning of the phosphorylation of the head domain? Nixon and Shea (1992) suggest a role in the early steps of the filament assembly. Otherwise, it might play a role in axonal transport of NF. NF have been shown in in vitro studies to be more dynamic structures than previously thought (Angelides *et al.*, 1989). Further recent fluorescent photobleach-recovery and biotin immunocytochemistry studies indicated directly that NF in the axons turned over within small area of the axoplasm, possibly by the mechanism of lateral and segmental incorporation of new subunits. These results suggest that although neurofilaments are

largely stationary, the moving units could be small fragments or oligomers (Okabe *et al.*, 1993).

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