Effects of phosphorylation of the neurofilament L protein on filamentous structures

**Shin-ichi Hisanaga,* Yasunori Gonda,†
Masaki Inagaki,† Atsushi Ikai,‡
and Nobutaka Hirokawa***

*Department of Anatomy and Cell Biology
Faculty of Medicine
University of Tokyo
Bunkyo-ku, Tokyo 113

†Laboratory of Experimental Radiology
Aichi Cancer Center Research Institute
Chikusa-ku, Nagoya 464

‡Laboratory of Biodynamics
Faculty of Science
Tokyo Institute of Technology
Nagatsuta, Midori-ku
Yokohama 227, Japan

Effects of phosphorylation of the neurofilament L protein (NF-L) on the reassembly system were studied by both sedimentation experiments and low-angle rotary shadowing. Bovine spinal cord NF-L was phosphorylated with 3–4 mol/mol protein by either the catalytic subunit of cAMP-dependent protein kinase or protein kinase C. Phosphorylated NF-L could not assemble into filaments. Phosphorylation by either cAMP-dependent protein kinase or protein kinase C inhibited the same step of the reassembly process. Phosphorylated NF-L remained as an 8-chain complex even in favorable conditions for reassembly. The extent of the effect of phosphorylation on the filamentous structure of NF-L was also investigated by using the catalytic subunit of cAMP-dependent protein kinase. The amount of unassembled NF-L increased linearly with increased phosphorylation in the sedimentation experiments. Structural observations indicated that 1 or 2 mol of phosphorylation is enough to inhibit reassembly and to induce disassembly, and the disassembly process was also observed. The filaments were shown to unravel with disassembly. Star-like clusters, which we reported as being the initial stage of reassembly, were also identified.

Introduction

Intermediate filaments (IFs)¹ have been thought to be a relatively stable cytoskeletal component as compared to other cytoskeletons such as microtubules and actin filaments (Lazarides, 1980; Steinert *et al.*, 1984). However, it has recently been suggested that IFs are dynamically reorganized particularly during mitosis and that this reorganization might be regulated by phosphorylation of IF proteins (Celis *et al.*, 1983; Evans and Fink, 1982; Evans, 1984, 1989; Chou *et al.*, 1989). Inagaki *et al.* (1987, 1988) first indicated that phosphorylation of desmin and vimentin by either cAMP-dependent protein kinase or protein kinase C inhibits their assembly in vitro (Inagaki *et al.*, 1987, 1988). Subsequent studies revealed that these phosphorylation sites are located at the amino-terminal head domain (Geisler and Weber, 1988; Evans, 1988; Ando *et al.*, 1989; Kitamura *et al.*, 1989). It has been suggested that the head domain plays a crucial role in filament formation (Traub and Vorgias, 1983). However, the exact extent of phosphorylation necessary for regulating the reassembly has not been elucidated yet.

IF shows a hierarchical subunit organization (Weber and Geisler, 1985; Steinert and Roop, 1988). Using the lowest subunit (NF-L) of a neurofilament (NF), we have recently presented a model for subunit arrangement and the reassembly process of NF in vitro (Hisanaga *et al.*, 1990; Hisanaga and Hirokawa, 1990). The smallest stable unit is a 4-chain complex in which two coiled-coil molecules align and register themselves in parallel. Two such 4-chain complexes construct an 8-chain complex by associating with each other in an anti-parallel manner with an overlap of ~50–55 nm (the entire length of the α -helix-rich central rod do-

¹ Abbreviations: IF, intermediate filament; NF, neurofilament; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid);

EGTA, glycoetherdiaminetetraacetic acid; DTT, dithiothreitol; TEE, 5 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, 0.1 mM EGTA and 0.1 mM DTT; 0.15NPM, 0.15 M NaCl, 20 mM PIPES (pH 6.8), 1 mM MgCl₂, 0.1 mM EGTA and 0.1 mM DTT.

main). The 8-chain complex is the building block of IF. Protofibrils are lengthened by end-to-end association of the 8-chain complexes. This elongation is promoted preferentially by NaCl. Four protofibrils make up the intermediate-sized filament in the presence of MgCl_2 . If the reassembly of NF proteins is also regulated by phosphorylation of the amino-terminal head domain as indicated in other IFs, it would be of interest to determine which step of the reassembly process described above is blocked by phosphorylation.

We approached these questions mainly by the method of low-angle rotary shadowing. This technique has previously been shown to be superior to other electron microscopic techniques for investigating the structure of IF, in the sense that glycerol decoration clearly reveals 22-nm repeats of beaded segments which represent the inherent secondary structure of the α -helix-rich central rod domain (Henderson *et al.*, 1982; Milam and Erickson, 1982). In this paper, we present evidence to show that 1 mol of phosphorylation per mole of NF-L is enough to disrupt the reassembly system of NF-L protein and also that phosphorylated NF-L remains as the 8-chain complex even under favorable conditions for reassembly. We also describe the intermediate structures observed when filamentous NF-L was induced to disassemble by phosphorylation.

Results

Phosphorylation of NF-L by cAMP-dependent protein kinase

The oligomeric form of NF-L was prepared by overnight dialysis against TEE at 4°C, and the filamentous form of NF-L was prepared by dialysis against 0.15NPM at 35°C for 3 h. The time course of phosphorylation of both forms of NF-L by the catalytic subunit of cAMP-dependent protein kinase is shown in Figure 1. Phosphorylation of oligomeric NF-L is represented by open circles. A maximum of 3–4 mol of phosphate was incorporated into 1 mol of NF-L (mol. wt 62 000) (Geisler *et al.*, 1985). The oligomeric form of NF-L was a better substrate for cAMP-dependent protein kinase than the filamentous form. When the same amount of enzyme was employed to phosphorylate the filamentous NF-L, it took longer to reach the plateau level (data not shown). It required a 3- to 3.5-fold amount of enzyme for the filamentous NF-L to show a similar phosphorylation curve to the one for the oligomeric form (closed cir-

cles). Even when the filamentous NF-L was used as the substrate, the final phosphorylation level was identical to that of the oligomeric NF-L.

Effect of phosphorylation on reassembly of NF-L in vitro

The effect of phosphorylation on the reassembly of NF-L was investigated by both a sedimentation experiment and electron microscopy. At the times indicated by arrows in Figure 1 (open circles) (these incubation times represent ~0, 1, 2, and 3 mol or a maximal incorporation of phosphate per mol of NF-L, respectively), NaCl and MgCl_2 were added to the reaction mixtures to make final concentrations of 0.15 M and 1 mM, respectively, and the reaction mixtures were incubated further for 1 h at 35°C to promote filament formation. After centrifugation at $90\,000 \times g$ for 1 h, supernatants and precipitates were run on SDS-PAGE and the amounts of assembled and unassembled NF-L were analyzed (Figure 2a). Before these sedimentation experiments, two control experiments were performed. First, the phosphorylation reaction was tested and it was observed that it did not proceed appreciably during the incubation period for reassembly. In the presence of 0.15 M NaCl (Figure 1, triangles), the catalytic subunit of cAMP-dependent protein kinase phosphorylated NF-L at the rate of only 0.35 mol/mol NF-L even after 2 h incubation (~1/10 of that in the absence of NaCl) (see also Inagaki *et al.*, 1988). Second, we found that under our centrifugation conditions only large aggregates were pelleted, not oligomeric forms. Neither the 4-chain complex, the 8-chain complex, nor the short intermediate-sized filament (Hisanaga *et al.*, 1990) was precipitated by centrifugation at $90\,000 \times g$ for 1 h, though we do not know the exact minimum size of the filaments that were precipitated.

Figure 2A shows that the amount of NF-L remaining in the supernatant increased as the degree of phosphorylation increased. The ratio of NF-L in the supernatant to that in the precipitate was estimated by densitometric scanning of the gel. The result is shown in Figure 3 (open circles). While >90% of NF-L precipitated before phosphorylation, >80% of NF-L remained in the supernatant after full phosphorylation. The amount of NF-L in the supernatant increased almost linearly with the increase of phosphorylation. At 2 mol of phosphorylation per mol of NF-L, 50–60% of NF-L was recovered in the supernatant.

Figure 1. Time course of phosphorylation of NF-L by the catalytic subunit of cAMP-dependent protein kinase. Phosphorylation of the oligomeric and filamentous NF-L is represented by open circles (○) and closed circles (●), respectively. Since the oligomeric NF-L was a better substrate for kinase, to obtain a similar time course of phosphorylation, 3.5-fold of kinase was used for phosphorylation of the filamentous NF-L. Triangles (△) indicate a phosphorylation of the oligomeric NF-L in the presence of 0.15 M NaCl. The phosphorylation rate was significantly reduced in the reaction mixture containing 0.15 M NaCl.

Structural observations were performed in parallel experiments. Unphosphorylated NF-L formed intermediate-sized filaments of various

length by incubation at 35°C for 1 h in the presence of 0.15 M NaCl and 1 mM $MgCl_2$ (Figure 4a). In this case, long filaments were rarely ob-

Figure 2. SDS-polyacrylamide gel electrophoresis to represent that phosphorylation inhibits the reassembly of NF-L and induces the disassembly of the filamentous NF-L. (A) Phosphorylation of the oligomeric form of NF-L. After 0, 5, 20, 60, and 120 min incubation with the catalytic subunit of cAMP-dependent protein kinase (at the times indicated by arrows in Figure 1), NaCl and $MgCl_2$ were added to the final concentration of 0.15 M and 1 mM, respectively, and the reaction mixtures were further incubated for 1 h at 35°C. After centrifugation at $90\,000 \times g$ for 1 h, the supernatants (s) and the precipitates (p) were subjected to 7.5% SDS-PAGE. (B) Phosphorylation of the filamentous NF-L. After 0, 5, 20, 60, and 120 min incubation with the catalytic subunit of cAMP-dependent protein kinase (at the times indicated by arrows in Figure 1), NaCl was added to the final concentration of 0.15 M and the reaction mixtures were chilled on ice. After centrifugation for 1 h at $90\,000 \times g$, the supernatants (s) and the precipitates (p) were subjected to 7.5% SDS-PAGE.

Figure 3. The ratio of the unassembled or the disassembled NF-L after phosphorylation. The amounts of NF-L recovered in the supernatant and in the precipitate were measured by densitometric scanning of the gels shown in Figure 2, and the ratio of NF-L in the supernatant to those in the precipitate was determined. The open circle is the phosphorylation of the oligomeric NF-L (Figure 2A) and the closed circle the phosphorylation of the filamentous NF-L (Figure 2B). The abscissa is expressed by the extent of phosphorylation (mol P/mol NF-L) which was calculated from Figure 1. The molecular weight of NF-L was considered to be 62 000.

served, in contrast to the filaments reassembled from NF-L by direct dialysis against 0.15NPM from a urea solution. Oligomeric structures were sometimes detected, perhaps due to both the low concentration of NF-L (0.15 mg/ml) and the fact that NF-L had been kept in the oligomeric form for phosphorylation reaction. NFs appeared to have a tendency, at least under our experimental conditions, to polymerize into longer filaments when they were brought into the polymerization condition gradually from the urea solution by dialysis.

The amount of intermediate-sized aggregates was significantly reduced and the number of oligomeric structures increased after only 5 min incubation with the catalytic subunit of cAMP-dependent protein kinase (~ 1 mol of phosphate

incorporation into 1 mol of NF-L) (Figure 4b). Twenty minute incubation (~ 2 mol of phosphate/1 mol of NF-L) kept the majority of NF-L in a state of an oligomeric, rod-like structure (Figure 4c), though a few small aggregates could still be identified. Some quantitative differences were recognized between the results of the sedimentation experiments and structural observations. Almost all the structures observed were oligomeric rodlets after 60 and 120 min incubation (>2.5 mol of phosphate/1 mol of NF-L) (Figure 4, d and e).

Effect of phosphorylation on the disassembly of NF-L filament

The effect of phosphorylation on the disassembly was also studied by the above method. At the times indicated by arrows in Figure 1 (closed circles), the reaction was stopped by adding 0.15 M NaCl and chilling the reaction mixture on ice. After centrifugation at $90\,000 \times g$ for 1 h, the extent of disassembly was estimated by measuring the amount of NF-L in the supernatant and in the precipitate. SDS-PAGE is shown in Figure 2B. Again, as phosphorylation proceeded, the amount of NF-L recovered in the supernatant increased. The ratio of NF-L in the supernatant to that in the precipitate was estimated by densitometric scanning of the gel (Figure 3, closed circles). The amount of NF-L in the supernatant increased with phosphorylation with an initial lag phase. When NF-L was maximally phosphorylated (~ 3.3 mol P/mol NF-L), 70% was recovered in the supernatant.

Structural changes caused by phosphorylation are shown in Figure 4, f–j. Before phosphorylation, NF-L was in the form of filaments showing 22 nm repeats, typical for IFs (Figure 4f). In general, as the phosphorylation proceeded, the number of long filaments decreased and the number of oligomeric structures increased concomitantly. Although many large aggregates were still observed at 1 mol of phosphorylation, many of them tended to unravel (Figure 4g). A small number of filamentous structures were still observed at 2 mol of phosphorylation (Figure 4h). At 3 mol of phosphorylation, almost all the structures observed were the oligomeric structures (Figure 4i), whereas only 50% of NF-L was recovered in the supernatant with the same degree of phosphorylation as by the sedimentation experiment (Figure 3, closed circles).

Higher magnification views of disassembling filaments

One mole of phosphate incorporation into NF-L appeared to be enough to disturb the filamentous organization. We observed various kinds of disassembling filaments when filamentous NF-L were incubated with the catalytic subunit of cAMP-dependent protein kinase for 5 min (~ 1 mol of phosphate per 1 mol of NF-L). Figure 5 shows higher magnification views of several examples of disassembling filaments. The disassembling filaments tended to unravel at their ends, in the middle, and sometimes along their entire length (Figure 5, a and b). Long protofibrils were also detected (arrow in Figure 5a). Twenty-two nanometers repeats of beaded segments were identified along protofibrils. Star-like clusters were also observed, as well as the initial stage of a filament assembly (the arrow in Figure 5c) (Hisanaga and Hirokawa, 1990). As phosphorylation proceeded, the length of the filaments decreased. Figure 5d depicts filaments which incorporated 2 mol of phosphate. Oligomeric rodlets appeared to be released directly from these short intermediate-sized filaments (arrows).

Which step of the reassembly process is inhibited by phosphorylation?

Detailed structural observations were performed to determine which stage of the filament formation is inhibited by phosphorylation. NF-L was phosphorylated by incubating with either the catalytic subunit of cAMP-dependent protein kinase or protein kinase C at 25°C for 2 h. Protein kinase C is already known to phosphorylate vimentin and desmin and inhibit their reassembly. cAMP-dependent protein kinase and protein kinase C incorporated 3.9 and 3.5 mol of phosphate into 1 mol of NF-L, respectively. These phosphorylated NF-L were dialyzed against a reassembly solution (0.15NPM) at 35°C for 3 h and the structures formed were observed. As described above, NF-L phosphorylated by either the catalytic subunit of cAMP-dependent protein kinase or protein kinase C did not reassemble into filaments, and only oligomeric structures were observed (Figure 6, a and b). Phosphorylation by either the catalytic subunit of cAMP-dependent protein kinase or protein kinase C seemed to arrest the reassembly reaction at the same stage.

Higher magnification views (Figure 6, c and d) show these oligomeric structures to resemble the 8-chain complex which we earlier reported

to be the real building block of IF. We showed in the previous paper (Hisanaga *et al.*, 1990) that the 8-chain complex is composed of two rods which are associated laterally and in an anti-parallel manner with an overlap of ~ 50 nm. As shown in Figure 6, c and d, we can recognize mostly overlapping regions, typical examples of which are indicated by arrows. The average length of the overlap region was 48 ± 7.8 nm ($n = 36$). This value supports the notion that the oligomeric structure observed here is the 8-chain complex.

Discussion

As reported with vimentin and desmin (Inagaki *et al.*, 1987, 1988), it was shown here that phosphorylation of NF-L by either cAMP-dependent protein kinase or protein kinase C inhibited the filament formation in vitro. Filaments were also induced to disassemble by the phosphorylation of NF-L. Major phosphorylation sites of NF-L were located in the amino-terminal head domain (Inagaki *et al.*, unpublished results), as the case with vimentin and desmin. These results suggest that the regulation of the assembly of NF-L by phosphorylation is similar to that of vimentin and desmin. To elucidate the assembly regulation of IF by phosphorylation, we focused on the following two questions in this paper: 1) how many moles of phosphate incorporation into NF-L are enough to inhibit reassembly and to induce disassembly of filaments and 2) which step of the in vitro reassembly process is inhibited by phosphorylation.

Phosphorylation keeps NF-L at the stage of the 8-chain complex

While the α -helix-rich central rod domain is required for maintaining coiled-coil interactions between subunit proteins, the amino-terminal head domain is considered to play a crucial role in filament assembly (Traub and Vorgias, 1983; Kaufmann *et al.*, 1985). However, it is not known at which step(s) of the reassembly process the N-terminal domain functions. Phosphorylation of the head domain of IF proteins is shown to result in the inhibition of reassembly. Then, phosphorylated NF which cannot assemble into filaments provide the means for determining the step(s). Rotary shadowing studies revealed that phosphorylated NF-L took on the form of an oligomeric, rod-like structure even in favorable conditions for reassembly. The same step was inhibited by phosphorylation caused by both cAMP-dependent protein kinase and protein ki-

Figure 4.

Figure 4. Rotary-shadowed electron micrographs of samples of NF-L with different degrees of phosphorylation. The oligomeric (a–e) and filamentous (f–j) NF-L were incubated with the catalytic subunit of cAMP-dependent protein kinase for 0 min (a and f), 5 min (b and g), 20 min (c and h), 60 min (d and i), and 120 min (e and j). In the case of the oligomeric NF-L, the phosphorylated samples were rotary shadowed after incubation at 35°C for 1 h in the presence of 0.15 M NaCl and 1 mM MgCl_2 . With the filamentous NF-L, phosphorylated samples were rotary shadowed after the addition of 0.15 M NaCl at the end of incubation. Bar represents 200 nm.

nase C. The oligomeric, rod-like structure was concluded to be the 8-chain complex because it was composed of two rods staggering with an overlap of ~ 50 nm. As mentioned above, our previous paper showed that the 8-chain complex is the real building block of NF (Hisanaga *et al.*, 1990).

A preliminary experiment of molecular weight measurement by sedimentation equilibrium tends to support this conclusion. The oligomeric form of phosphorylated NF-L showed a molecular weight of $\sim 370\,000$ which was very close to what we reported previously as the molecular

weight of the 8-chain complex (Hisanaga *et al.*, 1990).

We think that the N-terminal head domain is required for both longitudinal and lateral interactions between the 8-chain complexes. Phosphorylated NF-L did not form short intermediate-sized filaments even when the concentration of MgCl_2 was elevated to 10 mM (data not shown). MgCl_2 was shown to strengthen the lateral interactions between the 8-chain complexes (Hisanaga and Hirokawa, 1990). The functions of the N-terminal head domain seem to be common among IF proteins. We have also

Figure 5. Higher magnification views of the filamentous NF-L in the process of disassembly induced by phosphorylation. (a)–(c) are from replicas of the filamentous NF-L incubated with the catalytic subunit of cAMP-dependent protein kinase for 5 min (~ 1 mol phosphate incorporation into 1 mol NF-L). Unravelings were observed along the filaments and a long protofibril was also detected (arrow in a). Star-like clusters were also often identified (arrows in c). (d) is from a replica of the filamentous NF-L incubated with the catalytic subunit of cAMP-dependent protein kinase for 20 min. As the phosphorylation reaction proceeded, the length of the filaments decreased. Arrows indicate oligomeric rodlets that might have been released from the short filaments directly. Bar represents 100 nm.

observed that phosphorylated vimentin, desmin, and glial fibrillary acidic protein took on the shape of a rod-like structure similar to that observed with phosphorylated NF-L (Inagaki *et al.*, unpublished results).

One or two moles of phosphorylation is sufficient to disturb the reassembly system of NF-L

The maximum incorporation of phosphate was ~ 4 mol P/mol of bovine spinal cord NF-L by either the catalytic subunit of cAMP-dependent protein kinase or protein kinase C. When NF-L was phosphorylated to the maximum extent, it assumed the form of the 8-chain complex. Then how much phosphate incorporation is needed to inhibit the reassembly or to disassemble the

filaments? We analyzed this problem both structurally and biochemically. There were some quantitative differences between the sedimentation experiments and structural analyses. We think this might be due to some interactions between the 8-chain complexes composed of phosphorylated NF-L. Although it is clear that phosphorylated NF-L could not assemble into filaments, we cannot exclude the possibility that it self-interacts to some extent under the conditions favorable for polymerization. This was suggested by frequent observations of end-to-end association of the 8-chain complex on rotary-shadowed replicas. Rotary shadowing appears to reflect faithfully the phosphorylation-induced structural changes, although the effect of some shearing can not be avoided during the spraying of protein onto mica.

Figure 6. Rotary-shadowed electron micrographs of NF-L phosphorylated by the catalytic subunit of cAMP-dependent protein kinase and protein kinase C. Oligomeric NF-L was fully phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (a and c) or protein kinase C (b and d) as described in Methods. After dialysis against 0.15NPM, phosphorylated NF-L were rotary shadowed. Low (a and b) and high (c and d) magnification views are shown. Almost all structures observed were oligomeric rodlets. Similar structures were observed with NF-L phosphorylated by either the catalytic subunit of cAMP-dependent protein kinase or protein kinase C. The overlap region, which was indicated by arrows in c and d, was ~50 nm in length. Bars represent 200 nm (a and b) and 50 nm (c and d).

It should also be noted here that the degree of phosphorylation was only average. Each subunit in the 8-chain complexes was not necessarily phosphorylated to the same extent, and all the 8-chain complexes were not phosphorylated evenly. Uneven phosphorylation among

the 8-chain complexes might be particularly remarkable when the filamentous form of NF-L was used as a substrate, because the oligomeric structures were a better substrate of kinases than the filamentous NF-L. Disassembled 8-chain complexes might contain more phos-

phorylated sites than those remaining in the filaments.

Based on the structural analyses of the effect of phosphorylation on oligomeric NF-L, it is probably safe to conclude that 2 mol of phosphate incorporation is enough to inhibit the assembly. How about 1 mol of phosphorylation per mol of NF-L? Because the amounts of both intermediate-sized filaments and larger aggregates were greatly reduced after only 5 min incubation with the catalytic subunit of cAMP-dependent protein kinase, we think that 1 mol of phosphate incorporation is probably sufficient to inhibit the reassembly process. In the case of disassembly, a small amount of short filaments was still observed after 2 mol of phosphate incorporation. Three moles of phosphorylation per mole of NF-L turned almost all NF-L into the 8-chain complex. More phosphorylation appeared to be required to disassemble the filaments entirely. However, it also seemed certain that only 1 mol of phosphorylation could disrupt the integrity of the filaments.

Angelides *et al.* (1989) described in their recent paper that phosphorylated NF-L could not assemble but that phosphorylation of NF-L filaments appeared to stabilize them. The second of their observation is in contradiction with our results. Although it is difficult to comment on the discrepancy because no data were shown to support their statements, it is indisputable that the filamentous form of NF-L was a poorer substrate for kinases than the oligomeric form. More than a threefold amount of the catalytic subunit of cAMP-dependent protein kinase was required to phosphorylate the filamentous NF-L at the same rate as the oligomeric NF-L, and protein kinase C failed to phosphorylate the filamentous form of NF-L. It might be supposed that, depending on the phosphorylation conditions used, phosphorylation does not occur sufficiently to cause the disassembly of filaments.

In vivo function of phosphorylation of NF-L

It has been suggested that the phosphorylation of vimentin and desmin regulates the rearrangement of IF during mitosis (Celis *et al.*, 1983; Evans and Fink, 1982; Evans, 1984, 1989; Chou *et al.*, 1989). Neurons expressing NF, however, in contrast to cells expressing vimentin or desmin, do not proliferate. The question to arise then is what is the function of phosphorylation of the NF protein *in vivo*?

Almost all the reports about the phosphorylation of NF proteins dealt with the phosphor-

ylation of C-terminal domains (Julien and Mushynski, 1982; Jones and Williams, 1982; Carden *et al.*, 1985; Nixon and Lewis, 1986). The phosphorylation of the C-terminal domain of NF-M and NF-H subunits has been suggested to regulate the interactions between NF and other organelles (Hirokawa, 1982; Hirokawa *et al.*, 1984; Minami and Sakai, 1985; Eyer and Leterrier, 1988). NF-L is also reported to have the phosphorylation site(s) in the tail region, an area not mediated by the cAMP-dependent protein kinase (Sihag and Nixon, 1989). However, the functional meanings of phosphorylation of the C-terminal tail domain of NF-L, as well as of NF-M and NF-H remain subjects for future studies.

There has been no direct evidence to indicate the *in vivo* phosphorylation of the N-terminal domain of NF proteins, but *in vivo* cAMP-dependent phosphorylation of NF-L has been shown in the rat optic nerve by Sihag and Nixon (1989). They demonstrated that a phosphopeptide named L-2, which is readily phosphorylated by cAMP-dependent protein kinase *in vitro*, is also phosphorylated *in vivo* and is either not a substrate at all, or is a relatively poor substrate of calcium- and cyclic nucleotide-independent protein kinases and calcium- and calmodulin-dependent protein kinases. Considering those findings, our *in vitro* results would be the likely event *in vivo*, and it also seems probable that the first phosphorylation site would be important for both the inhibition of assembly and the disassembly of the filament.

Based on the observation that the phosphate group(s) on L-2 peptide is lost after neurofilament have advanced beyond the most proximal region of the axon (Sihag and Nixon, 1987), the same authors (1989) proposed that cAMP-dependent phosphorylation may be involved in facilitating the loading of neurofilaments onto the transport system. Together with our present data, at this stage then NF may necessarily be of oligomeric form. Possible roles of NF phosphorylation in axonal transport were also suggested in a study of Komiya *et al.* (1986). They detected a small portion of NF proteins migrating faster than the remaining bulk of NF in the rat dorsal root ganglion axon and suggested the faster migrating ones to be a soluble form of NF proteins (Tashiro and Komiya, 1989). Two pools of NF protein have also been described with the mouse retinal ganglion cell axon: the slowest phase of axoplasmic transport and the stationary network (Nixon and Logvinenko, 1986). The moving component was speculated to be the soluble form of NF (Hollenbeck, 1989).

Furthermore, it was recently shown by Angelides *et al.* (1989) using a fluorescence resonance energy transfer assay that NF is in fact a dynamic structure. If this is the case, the process of phosphorylation of the N-terminal domain will be the most likely candidate responsible for regulating the solubility of NF proteins during axonal transport.

Methods

Purification of proteins

NF-L was purified from bovine spinal cords by the method of Geisler and Weber (1981) with some modifications (Hisanaga and Hirokawa, 1988, 1989). Briefly, a crude extract of the spinal cord was incubated at 35°C for 30 min in the presence of 20% glycerol and centrifuged at $150\,000 \times g$ for 90 min to precipitate NF proteins. The precipitate was solubilized in a 6 M urea solution and NF-L was purified by column chromatographs on Whatman DE-52 and Mono-Q HR5/5 (Pharmacia, Uppsala, Sweden). Purified NF-L was concentrated by Centrifo CF25 (W. R. Grace & Co., Amicon Division, Danvers, MA) to 2–3 mg/ml and stored at –80°C.

The catalytic subunit of cAMP-dependent protein kinase was prepared from bovine heart by the method of Beavo *et al.* (1974).

Protein kinase C was prepared from rat brain by the method of Inagaki *et al.* (1985).

Phosphorylation of NF-L

NF-L (2 mg/ml) was dialyzed in advance against 5 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM dithiothreitol (DTT) (TEE) containing protease inhibitors (1 µg/ml leupeptin, 0.1 mM phenylmethylsulfonylfluoride) for phosphorylation of an oligomeric form of NF-L, or dialyzed against 20 mM PIPES (pH 6.8), 0.15 M NaCl, 1 mM MgCl₂, 0.1 mM EGTA, 0.1 mM DTT (0.15NPM) containing protease inhibitors for 3 h at 35°C for the phosphorylation of the filamentous form of NF-L. Both the oligomeric and filamentous forms of NF-L (0.15 mg/ml) were phosphorylated by incubation with 3 or 10.5 µg/ml catalytic subunit of cAMP-dependent protein kinase, 0.1 mM ATP, 0.3 mM MgCl₂, and 20 mM PIPES (pH 7.0) at 25°C or by incubation with 2.7 µg/ml protein kinase C, 0.1 mM ATP, 0.3 mM MgCl₂, 50 µg/ml phosphatidylserine, 2.5 µg/ml diacylglycerol, and 20 mM PIPES (pH 7.0) at 25°C (Inagaki *et al.*, 1988).

Electron microscopy

Low-angle rotary shadowing was performed by the method of Tyler and Branton (1980) as described previously by Hirokawa (1986) and Hisanaga and Hirokawa (1988). Phosphorylated NF-L was diluted to a protein concentration of ~50 µg/ml with 50% glycerol. Seventy-five microliters were sprayed onto a newly cleaved mica surface and rotary shadowed with platinum (2.5 nm) at an angle of 6° in a Balzers BAF301. Replicas were examined with a JOEL 1200EX electron microscope (JOEL, Tokyo, Japan). Magnification was calibrated as described previously (Hisanaga and Hirokawa, 1988).

Others

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) and stained

with Coomassie brilliant blue. The amount of NF-L was estimated by densitometric scanning of gels with a Dual-Wavelength Flying Spot Scanner CS-9000 (Shimazu, Osaka, Japan).

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