The Effects of Dephosphorylation on the Structure of the Projections of Neurofilament

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Carboxy-terminal tail domains of larger molecular mass subunits (NF-M and NF-H) of neurofilaments (NFs), which are the highly phosphorylated moieties, were observed as thin flexible filaments projecting from NF core filaments by rotary shadowing (Hisanaga and Hirokawa, 1988). Dephosphorylation of NFs has been suspected to affect the structures and the functions of the carboxy-terminal tail projections. We report here the effects of the dephosphorylation on the structure of NFs studied by electron microscopy. (1) The structures of carboxy-terminal tail projections after dephosphorylation were compared with those of the control NFs by low-angle rotary shadowing. This was examined with 2 samples; the isolated neurofilaments and the short filaments assembled from NF-H. Both the dephosphorylated NFs and the short filaments showed many projections laterally extending from core filaments similar to those observed in the control samples. (2) With respect to the structure of NF in physiological solution, the density of NFs in the precipitates was examined by thin-section electron microscopy. No difference in the density was noted between control and dephosphorylated NFs. (3) The ability to form cross-bridges *in vitro* was examined by quick-freeze, deep-etch electron microscopy. The structure and frequency of cross-bridges appeared to be similar in both control and dephosphorylated NFs. (4) Phosphate determination revealed that about 90% of the phosphate groups of NF-H subunit were removed by treatment with *E. coli* alkaline phosphatase. These results indicated that the dephosphorylation of NF did not affect the structure and the ability to form cross-bridges of the carboxy-terminal tail projections *in vitro*.

Neurofilaments (NFs) are one of the major cytoskeletal components in neuronal cells. NFs are usually composed of triplet polypeptides with different electrophoretic mobilities on SDS-PAGE. In the order of their molecular mass, they are designated NF-H, NF-M, and NF-L, respectively. All subunits are divided into 3 domains: the amino-terminal head domain, the α -helix-

rich rod domain, and the carboxy-terminal tail domain (Geisler et al., 1983, 1985). The α -helix-rich rod domain is responsible for the formation of the filaments. The carboxy-terminal tail domain has been suggested to constitute peripheral domains (Chin et al., 1983; Geisler et al., 1983, 1985; Julien and Mushynski, 1983). The difference in the molecular mass is due to the differences in the size of the carboxy-terminal tail domains. The carboxy-terminal tail domains of the higher-molecular-mass subunits (NF-H and NF-M) are suggested to be sites where NFs interact with each other and with other cytoplasmic organelles. We have recently demonstrated their structures both *in vivo* and *in vitro* by electron microscopy. It has been shown by the quick-freeze, deep-etch technique (QF-DE) that the NFs are extensively linked with each other by cross-bridges in the axon (Hirokawa, 1982). Immunocytochemical studies have revealed that several antibodies against the carboxy-terminal tail domains of NF-H and NF-M bind to peripheral regions of NFs (Willard and Simon, 1981; Sharp et al., 1982; Liem et al., 1985) and cross-bridges (Hirokawa et al., 1984). In a previous paper, we showed the structure of the carboxy-terminal tail domains of the isolated NFs by low-angle rotary shadowing (Hisanaga and Hirokawa, 1988). They were thin, flexible filamentous structures about 80 nm long and 3–4 nm wide, and projected from the core filaments at an interval of 22 nm.

The carboxy-terminal tail domains of NF-H and NF-M contain many phosphorylation sites (Jones and Williams, 1982; Julien and Mushynski, 1982, 1983; Carden et al., 1985). Major phosphorylation sites are shown to be located in a repetitive sequence motif of lysine-serine-proline (Geisler et al., 1987). Although there are some disagreements in the number of phosphorylation sites among reports, about 20–30 phosphorylation sites in NF-H and about 10 in NF-M are indicated. Phosphorylation of NF proteins is expected to play a crucial role in the function of the NF. NFs localize differently in neuronal cells depending on the extent of their phosphorylation (Sternberger and Sternberger, 1983). The removal of phosphate from the NF-M results in the dissociation of this protein from the core filament (Wong et al., 1984). Dephosphorylation suppresses the activity of NF-H to promote microtubule polymerization (Minami and Sakai, 1985).

Since the discovery and first use by Sternberger and Sternberger (1983) of monoclonal antibodies that recognize phosphorylated and unphosphorylated epitopes differentially, many reports have indicated that NFs present in the axon are highly phosphorylated, while those in the cell bodies and the dendrites are not (Bennett and Dilullo, 1985; Peng et al., 1986; Cohen et al., 1987; Hart et al., 1987; Lee et al., 1987; Oblinger, 1987). On the other hand, QF-DE electron microscopy indicates a

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different organization of NFs in axons, on the one hand, and cell bodies/dendrites, on the other (Hirokawa, 1982). NFs in the axon run parallel and are cross-bridged extensively. Those in the cell body tend to be randomly oriented, and the number of cross-bridges are reduced to about two-fifths of the axon. Those in the dendrite run parallel but are dispersed or exist as small fascicles, and the average frequency of cross-bridges is about three-fifths of the axon, although the frequency within a dendritic fascicle is similar to that in the axon.

On the basis of these findings, we thought that phosphorylation of NF subunits alters the conformation of the projections so as to form the cross-bridges, as suggested by several recent papers (Glicksman et al., 1987; Myers et al., 1987). Using low-angle rotary shadowing and QF-DE, we have been able to probe the effects of dephosphorylation on the structure of the projections. We found that the dephosphorylated NFs have similar structures of the projections to the phosphorylated NFs and that the cross-bridges are formed between the dephosphorylated NFs as well as the control NFs. We conclude from these studies that dephosphorylation affects neither the structure of the projections nor the ability to form the cross-bridges *in vitro*.

Materials and Methods

Preparation of the control and the dephosphorylated NFs. NFs were prepared from bovine spinal cords by gel filtration as described in the previous paper (Hisanaga and Hirokawa, 1988). Bovine spinal cords were homogenized in an equal volume of PEM [0.1 M PIPES (pH 6.8), 1 mM EGTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 μ g/ml leupeptin, and 0.5 mM phenylmethylsulfonylfluoride (PMSF)]. The homogenate was centrifuged at $55,000 \times g$ for 50 min, and the supernatant was dialyzed extensively against 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM DTT, and protease inhibitors (0.1 μ g/ml leupeptin and 0.1 mM PMSF). Dephosphorylation was carried out by incubating 0.8 ml of the dialyzed crude extract (~10 mg/ml) with 40–50 units of *E. coli* alkaline phosphatase (Wako, Osaka, Japan) for 5 hr at 35°C. The control was treated similarly without enzyme. At the end of the incubation, the control and the dephosphorylated NFs were applied on a Sepharose CL-4B gel filtration column (1.5 $\times$ 27 cm) and eluted with P_{20} EM (20 mM PIPES, 0.5 mM EGTA, 1 mM $MgCl_2$, 0.1 mM DTT, and protease inhibitors) containing 50 mM NaCl. The fractions eluted at a void volume were used as the isolated NFs.

Purification, reassembly, and dephosphorylation of NF-H. NF-H was purified by the method of Geisler and Weber (1981) with slight modifications (Hisanaga and Hirokawa, 1988). Porcine spinal cords were homogenized in PEM. The homogenate was centrifuged at $55,000 \times g$ for 50 min at 2°C. The supernatant was made 20% with respect to glycerol and incubated for 30 min at 35°C. NFs pelleted by centrifugation at $150,000 \times g$ for 90 min were dissolved in 10 mM sodium phosphate (pH 7.5), 1 mM EGTA, 6 M urea, 0.5 mM DTT, 0.1 μ g/ml leupeptin, and 0.1 mM PMSF (buffer A). After centrifugation at $48,000 \times g$ for 30 min, the protein solution was applied to a DEAE-cellulose column (Whatman DE-52) equilibrated with buffer A. Adsorbed proteins were eluted with a NaCl gradient (0–0.25 M) in buffer A. The fractions containing NF-H were collected and concentrated with centrifo CF-25 (W. R. Grace & Co. Amicon division, Danvers, USA) to a protein concentration of about 2 mg/ml. Concentrated NF-H (2 mg/ml) was polymerized by dialysis against P_{20} EM containing 0.15 M NaCl for 3 hr at 35°C. After further dialysis against 50 mM Tris-HCl (pH 8.5), 0.15 M NaCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM DTT, and protease inhibitors, dephosphorylation was carried out by incubating 0.2 ml of reassembled NF-H with 20 units of alkaline phosphatase at 35°C for 5 hr. The control sample was similarly treated without the enzyme. At the end of the incubation, the samples were applied on a Sepharose CL-4B column (1 $\times$ 25 cm) equilibrated with P_{20} EM containing 50 mM NaCl. Reassembled NF-H eluted at the void volume were used for observation.

Electron microscopy. Low-angle rotary shadowing was performed according to the method of Tyler and Branton (1980) as described previously (Hisanaga and Hirokawa, 1988). A small amount of NFs or the reassembled NF-H was diluted to adjust the protein concentration to

about 100 μ g/ml with 50% glycerol. Then, 50 μ l of the sample was sprayed onto a newly cleaved mica surface and, after drying under a vacuum at 5×10^{-6} torr, rotary-shadowed with platinum at an angle of 6° in Balzers BAF 301.

QF-DE electron microscopy was carried out as described previously (Hirokawa and Heuser, 1981; Hisanaga and Hirokawa, 1987). The control and the dephosphorylated NFs were pelleted by centrifugation at $30,000 \times g$ for 30 min. The precipitates were quick-frozen by contact with a pure copper block cooled by liquid helium. In some cases, to eliminate the artifacts by depositions of salt, the precipitates were fixed with 1% glutaraldehyde, 2% paraformaldehyde in PEM for 1 hr. After extensive washing with distilled water, they were quick-frozen as described above. The surface of the frozen samples were fractured at -196°C at a vacuum of below 2×10^{-6} , etched for 8 min at -95°C , and replicated with platinum at an angle of 24°.

For thin-section electron microscopy, the control and the dephosphorylated NFs were precipitated by centrifugation at $30,000 \times g$ for 30 min and then fixed with 1% glutaraldehyde and 0.2% tannic acid in PEM. After being postfixed in cold 2% OsO_4 in 0.1 M sodium cacodylate (pH 7.2), the samples were stained *en bloc* with 0.5% uranyl acetate for 2 hr. They were then dehydrated in ethanol and embedded in Epon 812. Thin sections were cut and double-stained with uranyl acetate and lead citrate.

Replicas and thin sections were viewed with a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan) operating at 100 kV. Magnification was calibrated by photographs of phyrophyllite (0.45 nm).

Phosphate determination. The control and the dephosphorylated NFs or NF-H were ashed over a strong flame after an addition of 10% $Mg(NO_3)_2 \cdot 6H_2O$ in ethanol and solubilized in 1.2 N HCl. Pi reagent [a mixture of 1 vol of 10% (wt/vol) ammonium molybdate and 3 vol of 0.2% malachite green] was added to this solution. After 5 min the absorbance was measured at 660 nm (Stull and Buss, 1977).

Others. SDS-PAGE was performed according to Laemmli (1970). Protein concentrations were determined by the method of Bradford (1976) using BSA as a standard. Measurements of the lengths of the lateral projections and the core filaments were carried out with a Nexus 641 image processor (Nexus, Tokyo) as described previously (Hisanaga and Hirokawa, 1988).

Results

Structure of the projections of control and dephosphorylated NFs

To observe the projections by low-angle rotary shadowing, NFs should be prepared delicately. We first carried out the dephosphorylation with the NF fraction isolated as previously. However, it was difficult to obtain convincing results because of (1) a contamination by particles of alkaline phosphatase and (2) a low concentration of NFs after a rechromatography to remove alkaline phosphatase, although similar results to those described below had been obtained. We therefore chose to dephosphorylate NFs in the crude extract. The crude extract of the spinal cord was extensively dialyzed against a Tris-buffered solution to increase pH and to remove nucleotides and other dialysable substrate for phosphatase. Figure 1A shows the time course of dephosphorylation. As already well known, NF-H and NF-M increased their electrophoretic mobility according to the extent of dephosphorylation. We used the mobility changes on SDS-PAGE as the index of dephosphorylation. In this condition, dephosphorylation was almost accomplished in 5 hr. Further incubation did not increase the electrophoretic mobilities. NFs dephosphorylated by incubating with alkaline phosphatase for 5 hr and the control NFs incubated without enzyme were isolated by Sepharose CL-4B, respectively. Gel filtration on Sepharose CL-4B alone was enough to enrich NF proteins for structural observation, although some contaminating proteins still remained in the fractions (Hisanaga and Hirokawa, 1988). Figure 1B shows SDS-PAGE of control and dephosphorylated NFs, which were used for electron microscopic observation.

Figure 2a is a rotary-shadowed image of a NF isolated from the crude extract. As shown in the previous paper (Hisanaga and Hirokawa, 1988), many projections about 80–90 nm long extended from the core filament. Figure 2, b, c, shows the control and dephosphorylated NFs, respectively. Both the control and dephosphorylated NFs appeared knobby compared with the untreated filaments shown in Figure 2a. Incubation at 35°C alone seemed to make the core filaments knobby. Some of NF-associated proteins might bind to, or some of the projections might retract on, the core filament during incubation at 35°C. Comparing control and dephosphorylated NFs, however, we did not detect structural differences in the projections. In both cases, the length of the projections was about 80–90 nm and the width about 4–5 nm. The average length of the projections was 87 ± 13 nm ($n = 119$) for control NF and 82 ± 12 nm ($n = 142$) for dephosphorylated NF, respectively. With respect to the frequency, although it was difficult to estimate correctly, no distinct difference was detected, at least within our measurements. The number of projections per 22 nm along NFs were 4.5 ± 0.5 ($n = 7$) for the control and 3.9 ± 0.4 ($n = 6$) for the dephosphorylated NFs, respectively.

Structure of the dephosphorylated filaments reassembled from purified NF-H

The projections of the isolated NFs were composed of NF-H and NF-M (Hisanaga and Hirokawa, 1988). To investigate the effect of dephosphorylation more clearly, we next examined structural changes after dephosphorylation by using the filaments reconstructed from NF-H, which is the most phosphorylated subunit and the most probable candidate of the cross-bridge. The time course of dephosphorylation of NF-H is shown in Figure 3a. Dephosphorylation was performed after the reconstruction of the filaments as described in Materials and Methods. The mobility of NF-H on SDS-PAGE increased according to dephosphorylation. A lower band in Figure 3A is *E. coli* alkaline phosphatase. Figure 3B shows the control (a) and the dephosphorylated (b) NF-H after gel filtration. These fractions were processed for rotary shadowing.

Rotary-shadowed images are shown in Figure 4. It is reported that long filaments are not formed from NF-H alone in the usual assembly condition (Geisler and Weber, 1981; Liem and Hutchison, 1982; Zackroff et al., 1982; Gardner et al., 1984). We also could not detect any long filaments having the same diameter as the isolated NFs. Instead, many short filaments, whose diameter was about $\frac{3}{4}$ of the native NFs, were reconstructed. Many projections—about 80 nm long and 4 nm wide—appeared from these short filaments (Fig. 4a). These sizes were almost the same as that of the native NFs. These projections seemed to correspond to the carboxy-terminal tail domains of NF-H. Even after dephosphorylation, the projections appeared as thin flexible structures extending from the short filaments (Fig. 4b). The dephosphorylated NF-H showed the projections with the same length and morphology as the control NFs. The average length of the projections was 75 ± 11 nm ($n = 170$) for the control and 78 ± 12 nm ($n = 156$) for the dephosphorylated filaments.

Packing density of the control and the dephosphorylated NFs in the precipitates

Rotary shadowing reveals the structure sprayed onto mica in the presence of a high concentration of glycerol. To eliminate possibilities that either the high concentration of glycerol or the adsorption to mica surface affects the structure of the projection,

Figure 1. Dephosphorylation of NF proteins. **A**, 7.5% SDS-PAGE of samples from a dephosphorylation time course experiment. Spinal cord extract (80 μ l, 10 mg/ml) was incubated with *E. coli* alkaline phosphatase (4 units) at 35°C for 0 hr (lane a), 0.5 hr (lane b), 1 hr (lane c), 2 hr (lane d), 4 hr (lane e), 7 hr (lane f). Aliquots were withdrawn at the times indicated above, and dephosphorylation was terminated by boiling in a Laemmli (1970) buffer. **B**, 7.5% SDS-PAGE of control (lane a) and dephosphorylated (lane b) NF after isolation through Sepharose CL-4B. Spinal cord extracts (0.8 ml, 10 mg/ml) were incubated with and without 46 units of alkaline phosphatase at 35°C for 5 hr. At the end of incubation, they were applied on Sepharose CL-4B column and NFs were isolated.

we next studied the structure in a physiological solution. By using negative staining electron microscopy, Carden et al. (1985) have already reported that the ultrastructural appearance of the NFs was unaltered by the extensive dephosphorylation. Unfortunately, however, the projections that we would have liked to observe were not detected by negative staining. Therefore, we decided to examine the packing density of NFs in the precipitates by thin-section electron microscopy. With the same method, Kim et al. (1979) clearly showed the difference of density between microtubules with and without MAPs. In the case of NFs, however, the dephosphorylated NFs pelleted in the packing density similar to the control NFs (data not shown).

Ability of the dephosphorylated projections of NFs to form cross-bridges

Results described above indicated that the structure of the projections was not altered by dephosphorylation. Next was the problem of whether the dephosphorylated projections are able to form the cross-bridges between NFs. The cross-bridges can be detected most clearly by QF-DE electron microscopy. Freshly isolated NFs were precipitated and processed for QF-DE as described in Materials and Methods. Cross-bridges between the isolated NFs formed even *in vitro* (Fig. 5a). The length of these cross-bridges was 20–50 nm and the width, 5–10 nm. These are the same sizes as observed in the axon. The frequency of the cross-bridges was not as abundant as in the axon. This may be because of the random orientation of NFs in the pellets. Although many proteins besides NF triplet proteins were still contaminated in the isolated NF fraction (Fig. 1B, lane a), the triplet proteins seemed to be sufficient to form the cross-bridges. The control NFs incubated without alkaline phosphatase are shown in Figure 5, b, c. The dephosphorylated NFs are shown in Figure 5, d–f. The fields where NFs ran relatively parallel were selected

Figure 2. Low-angle rotary shadowing electron micrographs of the non-treated (*a*), the control (*b*), and the dephosphorylated (*c*) NF. Numerous projections were observed extending laterally from the core filament. Arrowheads in *a* indicate the background-projections boundary. The core filament became knobby only after incubating at 35°C (*b* and *c*). No distinct difference was observed in the structure of the projection between the control and the dephosphorylated NFs. Scale bar, 100 nm.

to show the cross-bridges clearly. The cross-bridges were observed between the dephosphorylated NFs, as well as between the control NFs. The frequency of the cross-bridges appeared to be similar between the control and the dephosphorylated NFs. Although the fixed samples are shown in Figure 5, the same results were obtained with freshly prepared ones.

Determination of the phosphate content in the isolated NF and the short filament reconstructed from NF-H

There is an inconsistency about the remaining phosphates after dephosphorylation. In order to determine the extent of dephosphorylation in this condition, we measured the phosphate content in isolated NF or NF-H before and after dephosphorylation. Although only about half the phosphate groups were removed in the isolated NF fraction, about 90% of the phosphate groups were removed from the short filaments reassembled from NF-H by incubating with *E. coli* alkaline phosphatase (Table 1). The relatively large amount of phosphate remaining after dephosphorylation in the isolated NF fraction may be due to contributions by contaminating phosphopeptides and phospholipids. The amount of the phosphate groups in NF-H subunit measured was about 2- to 3-fold larger than the values reported previously (Julien and Mushynski, 1982; Wong et al., 1984;

Carden et al., 1985). Assuming a molecular mass of 110K for NF-H (Lees et al., 1988), however, the number of phosphate groups in NF-H would be 36 mol/mol NF-H; this value agrees relatively well with the number of putative phosphorylation sites (>40), the common amino acid sequence motif (lysine-serine-proline) (Geisler et al., 1987).

Discussion

One of the most interesting aspects of NFs is the phosphorylation. NFs display regionally different localization in neurons depending on their state of phosphorylation (Sternberger and Sternberger, 1982; Peng et al., 1986; Hart et al., 1987) and differential phosphorylation patterns during development (Dahl and Bignami, 1986; Dahl et al., 1986). Elucidation of the role of phosphorylation would lead to understanding the as yet unknown function of NF.

The effects of the phosphorylation of NF subunits have been thought to be mediated through the carboxy-terminal domains which are the multiple phosphate moieties in NF-M and NF-H. The different organizations of NFs in axons and cell bodies/dendrites suggested that phosphorylation particularly affects the structure of the cross-bridges. However, convincing results have not yet been obtained because the previous attempts to detect

Figure 3. Dephosphorylation of NF-H. *A*, 7.5% SDS-PAGE of samples from a dephosphorylation time course experiment. Short filaments reassembled from NF-H were dephosphorylated with alkaline phosphatase at 35°C for 0 hr (lane *a*), 0.5 hr (lane *b*), 1 hr (lane *c*), 2 hr (lane *d*), 4 hr (lane *e*), and 7 hr (lane *f*). Aliquots were withdrawn at the times indicated above, and dephosphorylation was terminated by boiling in a Laemmli (1970) buffer. The lower band is *E. coli* alkaline phosphatase. *B*, 7.5% SDS-PAGE of the control (*a*) and the dephosphorylated (*b*) NF-H after isolation through Sepharose CL-4B. Reassembled short filaments (0.2 ml, 1.8 mg/ml) was incubated with and without 40 units of alkaline phosphatase at 35°C for 5 hr. At the end of incubation they were applied on Sepharose CL-4B gel filtration column, and NF-H eluted at void volume was electrophoresed.

the structural changes were mainly performed using negative staining (Carden et al., 1985; Georges et al., 1986), which is unsuitable for observing the structure of the projections. Our previous work indicated that low-angle rotary shadowing is the only method capable of revealing the detailed structure of the projection (Hisanaga and Hirokawa, 1988) and QF-DE is the best way to observe the cross-bridges (Hirokawa, 1982; Hirokawa et al., 1984).

Unexpectedly, however, dephosphorylation did not affect the structure of the projections, at least *in vitro*. This was ascertained with 2 samples: freshly isolated NFs and short filaments reas-

Table 1. Phosphate content of NF and NF-H

	Phosphate content (nmol phosphate/mg protein)	
	Control	Dephosphorylated
Isolated NF	290 ± 27 (<i>n</i> = 4)	148 ± 26 (<i>n</i> = 4)
	Phosphate content (mol phosphate/mol polypeptide)	
	Control	Dephosphorylated
NF-H	36 ± 2 (<i>n</i> = 4) ^a	4 ± 2 (<i>n</i> = 4) ^a
	65 ± 4 ^b	8 ± 5 ^b

The phosphate contents before and after dephosphorylation were determined with the isolated NF and the filament reconstructed from NF-H. The numbers in parentheses represent the number of determinations. The listed values are means ± SD.

^a The number of the phosphate groups of NF-H assuming molecular mass of 110 kDa (Lees et al., 1988).

^b The number of the phosphate groups of NF-H assuming molecular mass of 200 kDa.

sembled from NF-H. We think that these results are not artifacts caused by high concentrations of glycerol and/or adsorption onto mica surface. The structural change of myosin by phosphorylation has been demonstrated by the same method (Onishi and Wakabayashi, 1982; Craig et al., 1983). The packing density of NFs in the precipitates suggested that dephosphorylation did not change the structure of the projections even in the physiological solution, although the entire length of the projection was not observed in this case.

To study the effect of dephosphorylation on the projection of NF-H we used the short filament reassembled from NF-H. NF-H is incapable of forming long filaments in the absence of NF-L (Liem and Hutchison, 1982; Zackroff et al., 1982; Gardner et al., 1984). Negative staining experiments show that by dialyzing against the assembly solution, NF-H assembled into about 7 nm curly structures (Gardner et al., 1984), short filaments 8–15 nm in diameter and 30–150 nm long (Tokutake et al., 1984), and 15–30 nm spherical and 40–400 nm long aggregates (Moon et al., 1981). Our rotary shadowing images coincide generally with these observations. By dialyzing against the reassembly

Figure 4. Low-angle rotary shadowing electron micrographs of the control (*a*) and the dephosphorylated (*b*) NF-H short filaments. The short filaments assembled from NF-H were dephosphorylated, gel-filtered, and rotary-shadowed as described in Materials and Methods. Note that many projections appeared extending from the short filaments that were 60–360 nm long and 15 nm wide. Scale bar, 100 nm.

Figure 5. QF-DE electron micrographs of NFs. NFs isolated through Sepharose CL-4B were centrifuged at $30,000 \times g$ for 30 min at 4°C. The precipitates were processed for QF-DE as described in Materials and Methods. *a*, The NFs isolated directly from spinal cord extract without incubation. *b–c*, Control NFs incubated at 35°C for 5 hr without enzyme; *d–f*, dephosphorylated NFs incubated with alkaline phosphatase at 35°C for 5 hr. Scale bar, 100 nm.

buffer, NF-H formed short filaments having a rather uniform diameter of about 15 nm. This value is about $\frac{3}{4}$ of that of isolated (21 nm) and reconstructed (19 nm) NFs (Hisanaga and Hirokawa, 1988). The large diameters are due to both decoration by glycerol and the thickness of the replica. Assuming the real width of NF to be 10 nm, $\frac{3}{4}$ of this value is 7.5 nm, which agrees well with the 7 nm reported by Gardner et al. (1984). In this case, too, the α -helix-rich rod domain seems to contribute to form the short filaments and the carboxy-terminal tail domain seems to constitute the projections as well in the intact NFs.

The cross-bridges were formed *in vitro* between NFs. The cross-bridges were observed with both fresh and fixed NFs. This eliminates the artifact by the deposition of salt. The sizes were very similar to those observed *in vivo*. Cross-bridges in both control and dephosphorylated NFs were about 15–60 nm long and 5–10 nm wide. These cross-bridges seemed to be comprised of the carboxy-terminal tail domain of NF-H and NF-M. This is supported by the fact that such apparent cross-bridges were not observed between filaments reconstructed from NF-L alone (Hirokawa et al., 1988). Whether a single projection can form a cross-bridge is unknown. That the cross-bridges were thicker than the projections may suggest a possibility that a single cross-bridge is composed of several projections. Although we think that NF triplet polypeptides are sufficient to form the cross-bridges, a possibility that proteins other than NF-M and NF-H

are required to form cross-bridge cannot be excluded on the basis of our experiments because many proteins besides triplet proteins were present in the NF fraction.

There are some contradicting reports about the phosphates that remained after the dephosphorylation by *E. coli* alkaline phosphatase. Carden et al. (1985) reported that *E. coli* alkaline phosphatase remove >90% of the phosphate groups from serine residue of NF-M and NF-H. In contrast, Georges et al. (1986) observed that bovine NF dephosphorylated under a similar condition still contained a considerable amount of phosphate (about a third of untreated NF). Our result—that most of the phosphate groups of NF-H were removed by the treatment with alkaline phosphatase—coincides with the result of Carden et al. (1985). Considering our data of the phosphate measurement, the results of structural observations described above can be put in the following way: Removal of most phosphate groups from NF-H did not affect the structure of the projections, at least, of NF-H. Assuming the molecular mass of NF-H to be 200 kDa, so as to facilitate comparison of our data with those of previous studies, the number of the phosphate groups in NF-H would be 65 mol phosphate/mol NF-H (Table 1), which is in between those (about 20–30 mol/mol NF-H) of Julien and Mushynski (1982), Wong et al. (1984), Carden et al. (1985), and Georges et al. (1986), and that (100 mol/mol NF-H) of Jones and Williams (1982). Taking the molecular mass of NF-H to be 110 kDa, as recently

estimated from the DNA sequence of NF-H (Lees et al., 1988), NF-H would contain 36 mol phosphate/mol polypeptide, which agrees relatively well with the number of the common motif of phosphorylation sites in NF-H (Geisler et al., 1987; Lees et al., 1988).

The present study indicates that dephosphorylation does not affect the structure of the projections, although it is possible that we overlooked small conformational changes. This brings us back to the initial question of why the organizations of NFs in the cell bodies and the dendrites differ from those in the axons. We think that there are 3 possible explanations. The first is simply the density of NFs. The number of NFs in the cell bodies and the dendrites is less than in the axons. Affinity may not be strong enough to form the cross-bridges between dispersed, randomly oriented NFs. The cross-bridges might be formed only after NFs enter the axon, where the concentration of NF might be sufficiently high for the projections to interact with the neighboring NFs. The second is that the dephosphorylated projections are bound by some NF-associated proteins that inhibit formation of cross-bridges within the cell bodies. Otherwise, some NF-associated protein(s), which localize mainly in the axon, may be required to form cross-bridges. The third is the content of NF-H and NF-M in the NFs. There are several different reports on the molar ratio of NF-L, NF-M, and NF-H in the NF fraction (Shekett and Lasek, 1980; Scott et al., 1985). One of the reasons for this discrepancy may be due to differences in starting materials for preparation of NFs, e.g., white matter-rich fraction vs gray matter-rich fraction.

Although the dephosphorylation at the carboxy-terminal tail domain of NF-M and NF-H did not change the structure of NFs at least *in vitro*, we think the phosphorylation may still play important roles in the function of NFs, such as regulation of the interactions of NFs with other cytoplasmic organelles and/or proteins. Further studies are necessary to understand the functional significance of NF phosphorylation.

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