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Phosphorylation of 68 kDa neurofilament proteins has no significant effect on their assembly

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Short communication

Abstract. Native low molecular weight neurofilaments (NF-L) from bovine spinal cord with original phosphate content of 0.4 moles of phosphate per 1 mol of protein were phosphorylated with cyclic AMP dependent protein kinase and protein kinase C. In a similar way recombinant mouse NF-L proteins which did not contain any phosphate were phosphorylated with the same enzymes in both, the assembled and disassembled forms. The final phosphate content in both types of NF-L proteins reached about 4 moles of phosphate per 1 mol of protein. This phosphorylation had no effect on the assembly of NF-L into filaments as observed by electron microscopy.

Key words: neurofilaments (NF-L), phosphorylation, assembly, electron microscopy

The eucaryotic cytoskeleton is composed of three types of filaments different in size, function and localization. Actin filaments are the smallest in diameter (about 8 nm), microtubules are the largest in diameter (about 25 nm). The third group of filamentous proteins is called intermediate filaments (IF) because they are of intermediate diameter (about 10-15 nm). These IF are not only more variable in their size and primary structure than other filaments, but they also differ in intracellular localization. According to the primary structure we can classify IF into three groups (Stewart 1993):

1. keratins;
2. desmin, vimentin, peripherin, glial fibrillary acidic protein; neurofilaments, α - internexin; nestin;
3. lamins.

Neurofilaments (NF) are neuron-specific IF and represent a major component of the neuronal cytoskeleton. They differ from the other IF by apparent content of many types of polypeptides, which differ in molecular size. They consist of the neurofilament triplet, three major polypeptides with apparent molecular weights of 200,000 Da (high molecular weight subunit; NF-H), 160,000 Da (middle molecular weight subunit; NF-M) and 68,000 Da (low molecular weight subunit; NF-L) (e.g. Liem et al. 1978). The subunit NF-L has been suggested to form the core of native neurofilaments and NF-H and NF-M coassemble with it making sidearms (Hisanaga and Hirokawa 1988). *In vitro* only NF-L can form IF-like structures after the assembly while NF-H or NF-M alone are forming only short filaments (Troncoso et al. 1989).

The view point of the statical function of NF should be abandoned at present. They apparently take part in dynamic processes of neuronal regeneration. Abnormal aggregation of NF was found *in vivo* as well as *in vitro* after intoxication with aluminium and other multivalent cations (Troncoso et al. 1990, Côte et al. 1993).

NF as well as the other IF are phosphorylated (Steinert et al. 1982). NF-H subunits contain more phosphate (about 100 mol PO_4/mol protein) than NF-M ones (about 25 mol PO_4/mol protein). NF-L subunits contain the smallest amount of phosphate groups - about 0.4 mol PO_4/mol protein (Jones and Williams 1982, Julien and Mushinski 1982).

As for the effect of phosphorylation on the assembly of NF-L proteins contradictory results were reported. Some authors (Gonda et al. 1990, Hisanaga et al. 1990, Nakamura et al. 1990, Mukai et al. 1996, Streifel et al. 1996) presented results which unequivocally showed that phosphorylation of NF-L proteins prevents their assembly and causes their disassembly. Even phosphorylation of serine in the position 55 was shown to be responsible for this phenomenon (e.g. Pant and Veeranna 1995). However, other authors report that the phosphorylation of NF-L proteins has small or no effect on the assembly of NF-L proteins (Guan et al. 1992, Giasson et al.1996, Gibb et al. 1996). Dephosphorylation of NF proteins by phosphatases had no effect on the assembly of these filaments (Georges et al. 1986, Hisanaga and Hirokawa 1989). Also importance of phosphorylation of serine in position 55 became questionable (Giasson et al.1996, Gibb et al. 1996).

The aim of the present paper is to contribute to the discussion of the problem of phosphorylation and its effects on the assembly and disassembly of NF-L.

A fresh or frozen bovine spinal cord was the starting material for NF-L proteins. The spinal cord was dissected about 30 min after death of the animal, meninges and blood vessels were stripped off and the cord was frozen and stored at -80°C or preferably immediately processed as described by Delacourte et al. (1980). This purification procedure of NF-L proteins included gel filtration on Sephadex G-25 and FPLC on Mono Q HR 5/5 anion exchange column.

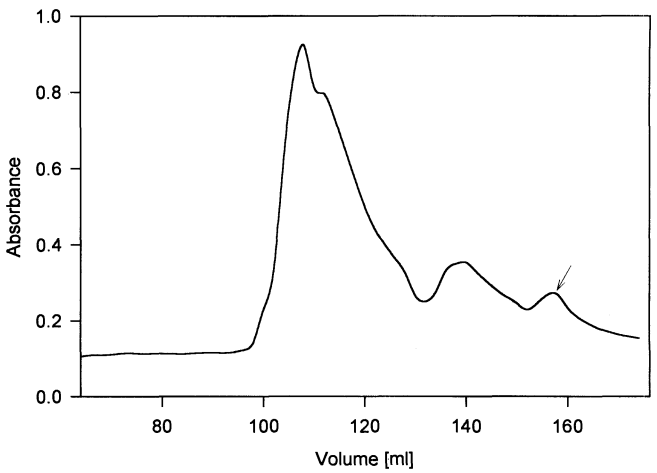

Fig. 1. Chromatography of native bovine NF proteins on Sephadex G-200. A sample (5 ml, NF proteins from 30 g of bovine spinal cord) was applied to a column (25 x 800 mm) of Sephadex G-200 (40-120 μm) equilibrated with buffer B (25 mM Na-phosphate, pH 7.5, 6 M urea, 1mM EGTA and 1 mM DTT). The elution was carried out with the same buffer at a flow rate of 12 ml/h. Fractions of 1 ml were collected. Absorbance was measured at 280 nm. Arrow indicates the peak of NF-L.

Alternatively the NF-L proteins were purified by gel filtration on a column (25 x 800 mm) of Sephadex G-200 (40-120 μm). Detailed description of conditions of this gel filtration is given in the legend to Fig. 1 which shows the elution profile. This way of purification of NF-L proteins has several advantages over the above mentioned methods. It is easier and does not depend on expensive instruments (FPLC) and, finally, the obtained preparation of NF-L proteins was "cleaner" than that obtained by the gel filtration on Sephadex G-25 and FPLC on Mono Q. The degree of purification of NF-L proteins was monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli 1970). The results are shown in Fig. 2.

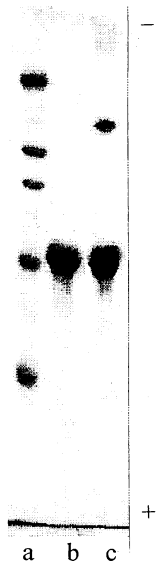

Fig. 2. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli 1970); a, standards of relative molecular weights [rabbit skeletal muscle myosin (200,000 Da), *E. coli* β-galactosidase (116,250 Da), rabbit muscle phosphorylase b (97,400 Da), bovine serum albumin (66,200 Da), hen egg white ovalbumin (45,000 Da)]; b, NF-L proteins purified by gel filtration on Sephadex G-200; c, NF-L proteins purified by gel filtration on Sephadex G-25 and by FPLC on Mono Q HR 5/5 anion exchange column.

NF-L proteins were isolated not only from the bovine spinal cord but were also obtained by the expression of mouse NF-L polypeptides in *Escherichia coli* (Studier and Moffat 1986) using the previously described expression vector (Gill et al. 1990, Heins et al. 1993). The cells (gift of Dr. U. Aebi, MIH Basel, Switzerland) were transformed with the above expression vector, cultured, induced (Heins et al. 1993) and resuspended in one tenth of the original volume in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.2, containing 170 mM NaCl, 1 mM DL-dithiothreitol (DTT) (buffer A) and lysed with a French press at a pressure of 20,000 p.s.i., the homogenate was centrifuged at 10,000 *g* for 15 min. at 4 °C. After 2 h of incubation at 37 °C the supernatant was centrifuged 20 min at 100,000 *g* at 25 °C. Pellet was suspended in 25 mM Na-phosphate, pH 7.5, 6 M urea, 1mM [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid (EGTA) and 1 mM DTT (buffer B), left overnight at 4 °C and treated in the same way as NF-L from the bovine spinal cord.

For phosphorylation of NF-L from the bovine spinal cord and recombinant mouse NF-L two types of kinases were used: cAMP-dependent protein kinase (catalytic subunit) - PKA (Promega, Madison, USA, 100 pmol of phosphate/min/μl) and protein kinase C - PKC purified as described by Uchida and Filburn (1984) with specific activity about 5 μmol of phosphate/min/μl.

Phosphorylation was performed on NF-L proteins obtained from the bovine spinal cord, recombinant mouse NF-L proteins as well as on filaments assembled from both of them. To assemble isolated NF-L before phosphorylation 100 μg/ml of NF-L proteins of both types (native or recombinant) were dialyzed for 3 h at 37 °C against buffer A, supplemented with phenylmethylsulfonyl fluoride (PMSF) to the final concentration of 1 mM. Disassembled (oligomeric form) NF-L proteins (native or recombinant) were prepared for phosphorylation by dilution (1/4, v/v) of isolated NF-L (100 μg/ml) with medium containing 5 mM Tris-HCl buffer, pH 8.3, 0.1 mM DTT and 1 mM PMSF. Both forms of both types of NF-L (0.25 μg in 15 μl) were then phosphorylated. First, the solutions were supplemented with $MgCl_2$ (2 mM), ATP (0.2 mM), [γ-^{32}P]ATP (3,000 Ci/mmol, 4μM) and then 1 μl PKA (or 0.6 μl PKC) was added. For treatment with PKC the reaction mixture was additionaly supplemented with $CaCl_2$ (0.2 mM), phosphatidylserine (50 μg/ml) and diolein (0.5 μg/ml). Mixtures were then incubated for 1 h at 25 °C. To assemble the filaments the both types of disassembled NF-L proteins were after phosphorylation dialyzed for 3 h at 37 °C against buffer A, supplemented with EGTA to the final concentration 1 mM.

The phosphate content in moles of PO_4 per mol of proteins was measured according to Jones and Williams (1982) and the amount of incorporated radioactive phosphate was measured radiographically with a liquid scintillation counter (Hollander and Bennett 1992). The amount of phosphate groups present naturally in NF-L proteins as well as in the assembled filaments together with the amount of phosphate groups found after the phosphorylation is summarized in Table I.

Possible changes in NF-L protein assembly after phosphorylation were followed by electron microscopy. For negative staining NF-L proteins were adsorbed to carbon coated 400 mesh/inch copper grids that were rendered hydrophilic by glow discharge (Aebi and Pollard 1987). An aliquot of the NF-L sample (5 μl) was placed

on the glow discharged grid, left for 50 s and the excess liquid of the sample was blotted. The grid was washed for 1-3 s with two drops of distilled water (remaining liquid was removed) before it was first washed and then incubated for 10 s in two drops of 0.75 % uranyl formiate pH 4.25 and the remaining liquid was removed not only with filter paper, but also by suction with a capillary applied to the edge of the grid. Electron microscopy was performed on Hitachi H-7 000 (Hitachi, Ltd., Tokyo, Japan) transmission electron microscope (TEM) operated on an accelerating voltage 100 kV using nominal magnification of x 50,000 .

No significant changes in assembly of NF-L proteins were observed after phosphorylation. These results were obtained with native bovine NF-L as well as with recombinant mouse NF-L (Fig. 3).

Mammalian NF-L proteins contain the lowest amount of phosphate (Jones and Williams 1982) compared with other NF. Apparently this naturally occuring low level of phosphorylation of NF-L cannot affect the assembly of these proteins. Therefore the recombinant mouse NF-L proteins which obviously do not contain any phosphate assemble in the same way as natural bovine NF-L proteins. Supplementary phosphorylation by protein kinases introduced the phosphate groups into recombinant mouse NF-L proteins and increased the phosphorylation level of bovine NF-L proteins. However, this increase of phosphate content to about 4 moles of phosphate per 1 mol of protein (Table I) was not sufficient to affect the assembly of these proteins. The phosphate content of NF-L after phosphorylation was similar to that already published (Julien and Mushynski 1982, Hisanaga et al. 1990). Apparently the NF-L proteins do not contain many accessible phosphorylation sites.

The isoelectric point of NF-L proteins lies in the acidic region (Hogue-Angeletti et al. 1982, Geisler et al. 1983) due to a high content of acidic amino acids, especially glutamic acid (about 20 %). This acidic character of NF-L protein can hardly be significantly changed by the relatively low level of possible phosphorylation. Electrostatic interaction which is definitely one of interactions in the assembly of protein chains into filamentous structures thus remains practically unchanged. This is the possible explanation for our finding of unchanged assembly of NF-L proteins after phosphorylation. On the other hand, phosphorylation near to arginine residues may affect the local charge in the N-terminal area of NF-L protein (Traub and Vorgias 1989). Phosphorylation of NF and other intermediate filaments has definitely some important physiological significance (Lewis and Nixon 1988, Brady 1993, Hall and Kosik 1993). The possibility remains that the phosphorylation which had no effect on NF-L assembly may affect the interaction of NF-L with other NF proteins.

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TABLE I

The phosphate content * in NF-L proteins and filaments assembled from them before and after phosphorylation with protein kinases

	natural phosphate content	phosphate content after the treatment with PKA	with PKC
NF-L protein from bovine spinal cord	0.5 ± 0.3	4.5 ± 0.5	3.5 ± 0.5
mouse recombinant NF-L protein	0	5.0 ± 0.5	4.0 ± 0.5
filaments assembled from bovine NF-L proteins	0.5 ± 0.3	2.0 ± 0.5	1.5 ± 0.5
filaments assembled from mouse recombinant NF-L proteins	0	2.5 ± 0.5	2.0 ± 0.5

*The phosphate content in moles of PO_4 per mol of proteins was measured according to Jones and Williams (1982) and the amount of the incorporated radioactive phosphate was measured radiographically with a liquid scintillation counter (Hollander and Bennett 1992).

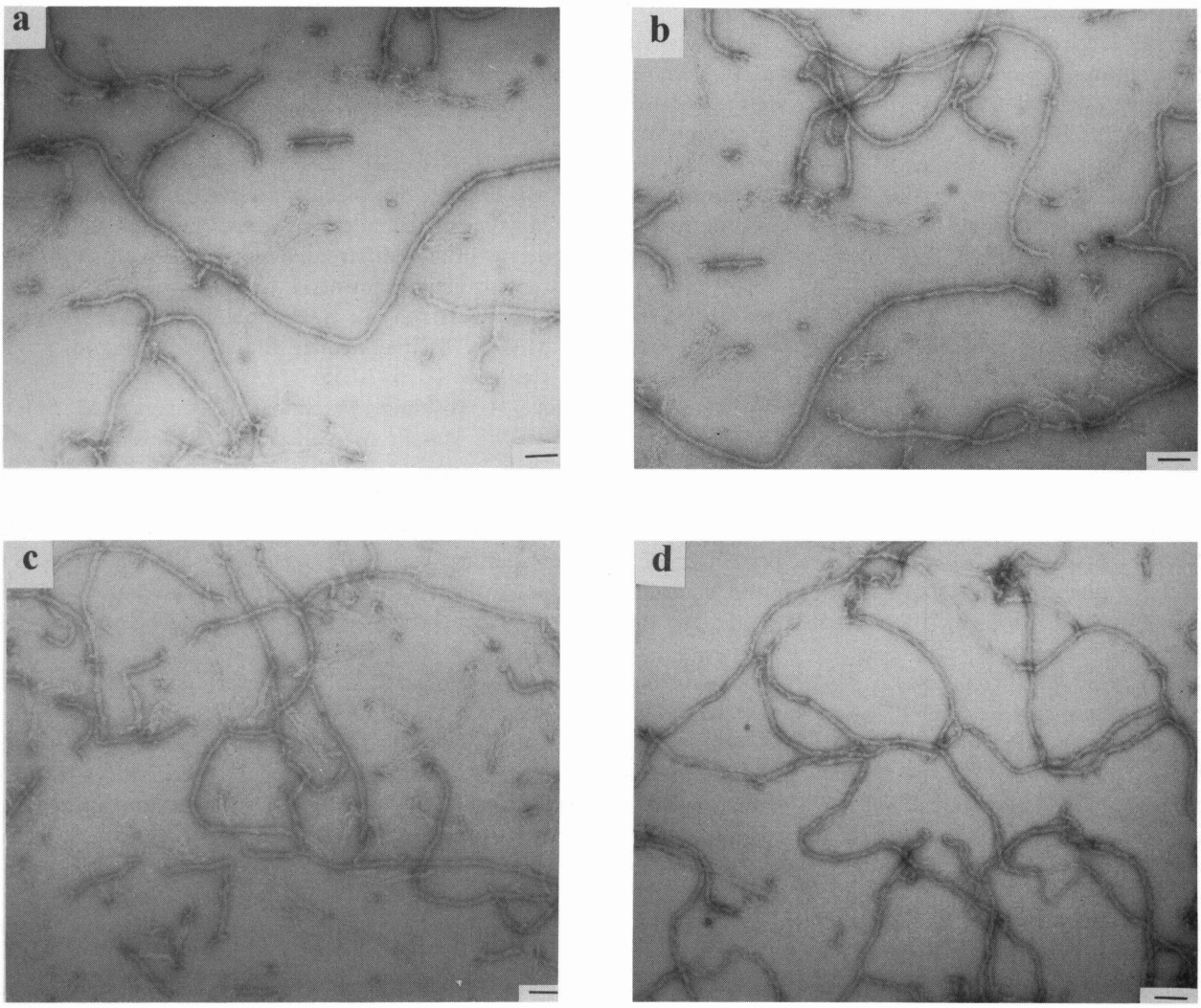

Fig. 3. Electron micrographs of NF-L proteins. Conditions of electron microscopy and phosphorylation are given in the text. NF-L proteins were assembled under standard filament forming conditions: dialyzed for 3 h at 37 °C against buffer A (50 mM MES, 170 mM NaCl, 1 mM DTT, pH 6.2) supplemented with EGTA to the final concentration 1 mM . Assembled NF-L were negatively stained with 0.75% uranyl formiate. Scale bar = 100 nm.; a, filaments assembled from bovine NF-L proteins; b, filaments assembled from phosphorylated bovine NF-L proteins; c, filaments assembled from mouse recombinant NF-L proteins; d, filaments assembled from phosphorylated mouse recombinant NF-L proteins.

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