Phosphatase and Carbocyanine Dye Binding Define Different Types of Phosphate Groups in Mammalian Neurofilaments

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The phosphorylation state of human and bovine spinal cord neurofilaments (NF) was studied by direct phosphate analysis and carbocyanine dye ("Stains-all") binding to NF polypeptides resolved on SDS-polyacrylamide gels. Electrophoretically purified NF-H (200 kDa), NF-M (160 kDa), and NF-L (68 kDa) of human origin contained 24, 18, and 4 mol phosphate/mol protein, whereas bovine NF contained 53, 23, and 5 mol phosphate/mol protein, respectively. Incubation of NF preparations with *E. coli* alkaline phosphatase removed about 55% of the phosphate from NF-H, about 30% of the phosphate from both human and bovine NF-M, but did not change the phosphate content of NF-L. This treatment also inhibited or substantially reduced the binding of electroblotted NF-H and NF-M to 2 anti-NF monoclonal antibodies known to recognize phosphorylated sites on projection side arms. "Stains-all" was found to be a very sensitive probe for detection of phosphorylated cytoskeletal proteins. Without the phosphatase treatment, NF and other phosphoproteins, MAP1, MAP2, tubulin, and tau, all bound the carbocyanine dye on SDS gels, forming blue dye-protein complexes. Measured densitometrically at 615 nm, the staining intensity (relative units/mol protein) was 9, 9, and 3 for human and 10, 13, and 6 for bovine NF-H, NF-M, and NF-L, respectively. NF-H bound the dye less efficiently than was expected from its phosphate content. After phosphatase treatment, NF-H, with half of its phosphate residues remaining, no longer formed blue complex with "Stains-all," the staining intensity of NF-M decreased by 20–40%, and the staining of NF-L was not changed. The results of dye binding, phosphate analysis, and immunostaining showed that there are at least 2 sets of phosphate groups in mammalian NF, which react differently with the phosphatase.

Neurofilaments (NF) are composed of 3 phosphoproteins, with molecular weights of about 200 kDa (NF-H), 160 kDa (NF-M), and 68 kDa (NF-L) that vary between species (Chiu et al., 1980; Lasek et al., 1985). NF-H has been shown to contain the highest amount of phosphate and NF-L the least (Jones and Williams, 1982; Julien and Mushynski, 1982; Wong et al., 1984; Carden

et al., 1985). Phosphorylation of NF proteins has been demonstrated *in vivo* (Eagles et al., 1981; Julien and Mushynski, 1981; Honchar et al., 1982; Shecket and Lasek, 1982). However, only limited information is available regarding the enzymes involved in this process. *In vitro* studies showed that phosphorylation of NF could be catalyzed by cAMP, Ca²⁺-independent protein kinases (Runge et al., 1981; Julien et al., 1983; Toru-Delbauffe and Pierre, 1983), or by calmodulin-dependent protein kinases (Vallano et al., 1985). These enzymes were detected in NF-enriched preparations. Dephosphorylation of NF proteins in tissue sections or test tubes has been achieved by incubation with alkaline phosphatases from *E. coli* or calf intestine (Julien and Mushynski, 1982; Sternberger and Sternberger, 1983; Carden et al., 1985; Georges et al., 1986). Upon dephosphorylation, there is an increase in the electrophoretic mobility of NF-H and NF-M, but not of NF-L.

Immunocytochemical studies demonstrated that certain antigenic determinants of phosphorylated NF are expressed in axons, but not in neuronal cell bodies (Sternberger and Sternberger, 1983). The significance of such "heterogeneity" is uncertain. The axon-specific anti-NF antibodies were recently shown to recognize filaments that had accumulated abnormally in neuronal cell bodies after exposure to some neurotoxins (Troncoso et al., 1985), and they also reacted with neurofibrillary tangles found in Alzheimer's disease (Sternberger et al., 1985; Yen et al., 1985; Cork et al., 1986) and other neuropathological conditions (Nakazato et al., 1984). Furthermore, many tangle-reactive anti-NF antibodies were recently found to recognize the heat-stable, phosphorylated microtubule-associated proteins tau and MAP2 (Ksiezak-Reding and Yen, 1987; Ksiezak-Reding et al., 1987; Nukina et al., 1987). These observations have led to the hypothesis that the phosphorylation state of NF or other associated cytoskeletal components may affect the distribution or movement of NF. An understanding of the phosphorylation of NF at different subcellular locuses, in normal versus pathological conditions and in different species, would be important for studying the role of NF phosphorylation.

The phosphate content of NF triplet proteins from animal tissues has been studied by several laboratories (Jones and Williams, 1982; Julien and Mushynski, 1982; Wong et al., 1984; Carden et al., 1985; Georges et al., 1986). No information, however, is available regarding the state of phosphorylation of NF derived from human tissues. Such data may provide some clue as to why neurofibrillary degeneration of the Alzheimer's type occurs only in humans. In the following studies, 2 methods were used to analyze the state of phosphorylation of human NF proteins. The results are compared with those obtained from nonhuman tissues.

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Materials and Methods

Preparation of NF and microtubules. Human spinal cords (4–14 hr postmortem) and bovine spinal cords obtained from a local slaughterhouse were stored at -80°C before use. The NF-enriched samples were prepared by the method of Chiu and Norton (1982). The resulting pellet was suspended in a buffer containing 0.1 M Tris-HCl, pH 8.0, 1 mM MgSO_4 , 1 mM EGTA, 0.1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride, or in a similar buffer without MgSO_4 , EGTA, and EDTA. NF (3 mg of protein) were incubated with 50 U/ml of *E. coli* alkaline phosphatase (Sigma, type III) for 16 hr at 37°C . The addition of MgSO_4 , EGTA, and EDTA to the incubation medium improved the reaction to some extent. A control sample was incubated similarly without the phosphatase. The enzyme-treated and control samples were centrifuged for 10 min at $10,000 \times g$. In some experiments, the NF preparation was suspended in the above buffer (adjusted to pH 6.5) and incubated with neuraminidase (Sigma, type X), 1 U/ml/3 mg NF protein, in the same conditions as for the alkaline phosphatase.

The microtubule fraction was prepared from bovine brain by double cycle of polymerization, according to the procedure reported by Murphy (1982) with minor modifications (Ksiezak-Reding and Yen, 1987). Protein content was determined by the method of Lowry et al. (1951).

Gel electrophoresis and staining with "Stains-all." SDS-gel electrophoresis was performed on 7.5 or 10% polyacrylamide gels (1.5 mm thick) using the discontinuous system of Laemmli (1970). To stain phosphoproteins on gels, we used a modification of the procedures described by Green et al. (1973) and Campbell et al. (1983). The gels were fixed overnight in 25% methanol/10% acetic acid and then washed for 3 hr in 25% methanol (3 changes) and for 2 hr in 25% methanol/30 mM Tris-HCl, pH 8.8 (2 changes). The gels were stained overnight (in the dark) with a freshly prepared cationic carbocyanine dye solution containing 25% methanol, 7.5% formamide, 30 mM Tris-HCl, pH 8.8, and 0.0025% 1-ethyl-2-/3-(1-ethylnaphto/1,2d/thiazolin-2-ylidene)-2-methyl-propenyl/-naphto/1,2d/thiazolium bromide (the cationic carbocyanine dye; Sigma, "Stains-all"). The stained gels were washed for 1 hr in 25% methanol (2 changes) and were photographed through a red filter. The gels were completely destained by repetitive washes as above, then stained with Coomassie blue or Fast green as described earlier (Gorovsky et al., 1970). Fast green was more reliable than Coomassie blue in the quantitative analysis of NF proteins (Chiu and Norton, 1982). Gels stained with "Stains-all" and those stained with Fast green were scanned on a Gilford spectrophotometer at 615 and 660 nm, respectively. The area under each peak was determined by an integrator or by weighing the paper. Ovalbumin was used as a standard in the quantitative analysis of the Fast green-stained proteins.

Determination of phosphate content of NF proteins. The individual NF polypeptides used for phosphate analysis were separated by SDS-PAGE. The respective protein bands were identified by incubating the gels with 4 M sodium acetate for 30 min (Higgins and Dahmus, 1979). The excised gel strips were washed in distilled water (2×10 min), cut into small pieces, and subjected to electroelution in an ISCO electrophoretic concentrator (Model 1750; ISCO). The inner and sample chambers contained 2.5 mM Tris-base, 19.2 mM glycine, and 0.05% SDS, and the electrode chamber contained 25 mM Tris-base, 192 mM glycine, and 0.05% SDS. Electroelution was carried out for 4–5 hr at room temperature. The 100–250 μl aliquots of the eluate were dried and ashed twice with 30 μl of 10% $\text{Mg}(\text{NO}_3)_2 \times 6 \text{H}_2\text{O}$ in 95% ethanol (Ames, 1966). The residues were dissolved in 0.5 N HCl, boiled for 20 min, and the samples were analyzed for inorganic phosphate content by the microadaptation of the malachite green method of Itaya and Ui (1966). A standard solution of inorganic phosphate (Sigma) was also subjected to the ashing procedure. Respective controls were performed for ashing efficiency (with ovalbumin) and for interference of the elution buffer and magnesium nitrate.

Immunoblotting. NF proteins separated on SDS gels were electrophoretically transferred to nitrocellulose paper (Towbin et al., 1979). Immunoblotting was carried out with 2 well-characterized monoclonal anti-NF antibodies, AD10 and Ab18, as reported previously (Yen et al., 1985; Ksiezak-Reding and Yen, 1987).

Results

Binding of "Stains-all" dye to NF and other phosphoproteins

The NF-enriched samples prepared from human and bovine spinal cords, in addition to NF triplet proteins, contained glial

filament proteins and other polypeptides. In most of the preparations, only NF proteins were stained blue by the cationic carbocyanine dye (Fig. 1). Other protein bands were stained red. The intensity of the blue staining of NF proteins correlated well with the protein content. In the microtubule fraction, several protein bands also formed blue complexes with the dye (Fig. 1). These included microtubule-associated protein (MAP)1, MAP2, tau proteins, tubulin, and proteins that comigrated with NF polypeptides on SDS gels.

Decreased binding of "Stains-all" and anti-NF antibodies to phosphatase-treated NF

The electrophoretic mobility of NF-H and NF-M obtained from either human or bovine spinal cord was changed after incubation of the NF preparation with phosphatase (Fig. 2A). For the human, the apparent molecular weights of NF-H and NF-M were reduced to about 180 and 150 kDa, respectively, and for bovine, to 170 and 150 kDa, respectively. The molecular weight of NF-L from both species, by contrast, was not altered. Our results are comparable to those reported to other investigators (Carden et al., 1985; Kaspi and Mushynski, 1985).

NF-H in the phosphatase-treated NF preparations from both species was no longer stained blue by "Stains-all"; instead, it was stained red (Fig. 2B). NF-M retained the ability to form blue complex with the dye, but the intensity of staining was decreased as compared to that of the untreated samples. No apparent changes were detected in the staining of NF-L by the dye.

Electroblotted NF-H and NF-M from untreated preparations reacted with 2 anti-NF monoclonal antibodies, AD10 and Ab18 (Fig. 2C). These antibodies have been previously found to recognize phosphorylated sites on NF-H and NF-M (Ksiezak-Reding and Yen, 1987). Treatment of NF preparation with phosphatase blocked the binding of AD10 to NF-H and NF-M. It also reduced the binding of Ab18 to NF proteins.

Quantitative analysis of phosphate content and "Stains-all" binding in NF proteins

Quantitative phosphate analysis of human and bovine NF proteins showed that NF-H and NF-M in both species were more phosphorylated than was NF-L (Table 1). Furthermore, bovine NF-H was found to contain twice as much phosphate (53 mol) per mol protein as human NF-H (24 mol). The species differences were not so striking in the other two NF proteins. Bovine phosphate content of NF-M (23 mol) was only moderately higher than human (18 mol). Bovine and human NF-L contained similar amounts of phosphate—5 and 4 mol, respectively.

The phosphatase treatment of NF preparations from both species reduced the amount of phosphate by 50–60% in NF-H and by 28–30% in NF-M (Table 1). The phosphate groups in NF-L were apparently insensitive to the enzyme under these conditions.

By comparing the intensity of staining of NF proteins resolved on SDS gels with the carbocyanine dye and Fast green it became apparent that there was a linear relationship between "Stains-all" binding and the amount of protein (Fast green) for each individual NF polypeptide (Fig. 3). Since the ratio of binding (in arbitrary units) of "Stains-all" to Fast green was always higher than 1 for the untreated NF polypeptides, "Stains-all" appeared to be a more sensitive probe in detecting the phosphoproteins than was Fast green. In human preparations, the ratios of "Stains-all" binding to that of Fast green were 1.3, 1.7,

Figure 1. Coomassie blue (A) and "Stains-all" (B) double staining of NF preparations from (a) human or (b) bovine spinal cords, (c) microtubule fraction from bovine brain, and (d) heat-stable microtubule fraction from bovine brain. SDS-polyacrylamide gels, 7% (a, b) or 10% (c, d) were first stained with "Stains-all," and a photograph was taken through a red filter. Gels were then destained and stained again with Coomassie blue. The location of NF triplet proteins, as well as of MAP1, MAP2, tau and beta-tubulin (T) is indicated.

and 1.3 for untreated NF-H, NF-M, and NF-L, respectively, and 0.1, 1.0, and 1.5 for the respective phosphatase-treated NF. In bovine preparations, the ratios of "Stains-all" binding to that of Fast green were 1.6, 2.6, and 2.5 for the untreated NF triplet,

respectively. After phosphatase treatment, they decreased to 0, 2.1, and 2.5 for the respective NF.

The binding of "Stains-all" (relative units) per mol of each of the NF triplet proteins (Table 2) showed that, in analogy to

Figure 2. Coomassie blue (A) and "Stains-all" (B) double staining, and immunoblots (C) of control and phosphatase-treated NF preparations resolved on 10% SDS gels. In A and B, double staining was performed as described in Figure 1 for (a) untreated or (b) phosphatase-treated NF preparation from human, and (c) untreated or (d) phosphatase-treated NF preparation from bovine. C, Electrophoretically transferred NF proteins of bovine from (a) untreated or (b) phosphatase-treated NF preparations stained with AD10, and (c) untreated or (d) phosphatase-treated NF preparations stained with Ab18. The locations of NF-H, NF-M, and NF-L are indicated. Marks at right (A) correspond to standard proteins of molecular weight (from top) 205 kDa (myosin), 116 kDa (beta-galactosidase), 97 kDa (phosphorylase B), 66 kDa (albumin, bovine) and 45 kDa (albumin, egg).

Figure 3. The relation between "Stains-all" binding (blue complex) and amount of protein (Fast green binding) in triplet NF polypeptides from human spinal cord. NF-H (A), NF-M (B), and NF-L (C) were resolved on SDS gels, stained with "Stains-all" or Fast green, and scanned at 610 or 660 nm, respectively. Areas under each peak were measured and expressed in arbitrary units. Untreated (open circles) and phosphatase-treated (filled circles) NF preparations. Each point represents an individual measurement.

Table 1. Phosphate content of human and bovine neurofilament triplet polypeptides

Species	NF triplet polypeptide	No treatment (mol P/mol protein)	Alkaline phosphatase treatment (mol P/mol protein)	Phosphate groups removed (mol P/mol protein)
Human	NF-H	24 ± 4	12 ± 3	12
	NF-M	18 ± 2	13 ± 2	5
	NF-L	4 ± 1	4 ± 1	0
Bovine	NF-H	53 ± 8	21 ± 3	32
	NF-M	23 ± 1	16 ± 3	7
	NF-L	5 ± 1	4 ± 1	1

Molecular weight of NF triplet polypeptides was assumed to be equal in both species: 200 kDa (NF-H), 160 kDa (NF-M), and 68 kDa (NF-L). Phosphate content was measured in ashed samples of electrophoretically purified NF. Conditions for phosphatase treatment and other details in Materials and Methods. Mean values ± SEM of 3–6 experiments are presented as mol P/mol protein.

Table 2. Carbocyanine dye "Stains-all" binding in human and bovine neurofilament triplet polypeptides

Species	NF triplet polypeptide	No treatment (dye binding/mol protein)	Alkaline phosphatase treatment (dye binding/mol protein)	Phosphate groups removed (dye binding/mol protein)
Human	NF-H	9 ± 1	0.6 ± 0.5	8
	NF-M	9 ± 2	5 ± 1	4
	NF-L	3 ± 0.3	3 ± 1	0
Bovine	NF-H	10 ± 1	0	10
	NF-M	13 ± 3	11	2
	NF-L	6 ± 1	6	0

Molecular weight of NF triplet polypeptides was assumed to be equal in both species (see legend to Table 1). Results are expressed in arbitrary units of dye binding/mol protein using Fast green-stained ovalbumin as a protein standard (see Fig. 3 for comparison). Values (dye binding/mol protein) are means ± SEM for 9–10 gel scans (human) and means ± range from 2 scans or the results of 1 scan only (bovine). Conditions for phosphatase treatment and other details in Materials and Methods.

direct phosphate analysis, more blue complex is formed per mol of NF-H or NF-M than per mol of NF-L. However, in contrast to the direct phosphate analysis, NF-H and NF-M from human or bovine preparations seem to bind the dye equally in spite of a 1.5–2-fold difference in the phosphate. It appears that NF-H does not bind the dye as efficiently as would be expected from its phosphate content.

The formation of blue complex between NF polypeptides and the carbocyanine dye was reduced by the treatment of NF with phosphatase (Table 2; Fig. 3). The changes correlated to some extent with a reduction of phosphate content. However, the dye-binding method underestimated the quantity of phosphate residues removed from high-molecular-weight NF proteins. This was clearly evident in NF-H from bovine or human preparations.

The results of carbocyanine dye binding showed that this dye is more sensitive than Fast green in detecting untreated NF polypeptides, and that the "Stains-all" binding technique is more suitable for qualitative than for quantitative analysis of phosphate groups in NF polypeptides.

Discussion

This study shows that NF-H and NF-M prepared from bovine tissue contain more phosphate per mol of protein than does NF-L. These results are in agreement with that reported earlier (Wong et al., 1984; Minami and Sakai, 1985). By comparing human and bovine NF, it is apparent that, with the exception of NF-L, bovine NF proteins contain larger amounts of phosphate than human NF. This difference is not likely due to the postmortem removal of some phosphate groups in human NF, since the electrophoretic mobility of NF polypeptides obtained from fresh postmortem material was similar to that obtained after a longer delay. Furthermore, phosphate residues in isolated NF seem to be relatively stable, since the difference between bovine and human phosphate content of NF polypeptides persisted after 16 hr of incubation at 37°C. The phosphate content of human NF triplet proteins presented here (24, 18, and 4 mol phosphate/mol protein) is similar to that obtained from rat or porcine NF triplet proteins (Julien and Mushynski,

1982; Georges et al., 1986), which have about 22, 10, and 3 mol phosphate/mol protein.

With regard to the phosphate content of bovine NF proteins, the values reported by different investigative groups differ (Jones and Williams, 1982; Wong et al., 1984; Carden et al., 1985; Minami and Sakai, 1985). For NF-H, they vary between 25 and 104 mol phosphate/mol protein (200 kDa). For NF-M (160 kDa), they range from 10 to 26, and for NF-L (68 kDa) from 0.4 to 3. The high variability of phosphate data between laboratories may reflect differences in the assays employed. It may also be affected by the procedures used in purifying NF proteins.

Incubation of NF proteins with alkaline phosphatase (*E. coli*) did not remove all the phosphate groups. In both human and bovine NF, dephosphorylation was more extensive for NF-H than for any other NF protein. In NF-H, up to 60% of the phosphate residues could be removed, in NF-M about 30%, whereas in NF-L only very little phosphate (if any) could be removed by the enzyme. Treatment of porcine NF with phosphatase (calf intestine or *E. coli*) has been shown to result in a reduction of 63, 50, and 31% of phosphate content from the respective NF triplet (Georges et al., 1986). A near complete (more than 90%) dephosphorylation of NF proteins by phosphatase was observed by Carden et al. (1985). The phosphate content of their untreated NF proteins, however, was much lower than that found by others.

The variable effect of phosphatase on different NF polypeptides could be due to several factors, e.g., a spatial obstruction or an intrinsic resistance of some phosphate group to the enzyme. Such resistance in ovalbumin is known to be due to the specific amino acid sequence surrounding the P-seryl residues (Flavin, 1954). Phosphate groups resistant to the phosphatase treatment have also been found in some cytoskeletal proteins. Incubation of MAP2 with *E. coli* alkaline phosphatase removed only 40–50% of the phosphate found in that protein (Selden and Pollard, 1983). Other phosphatases, from brain (calcineurin) or smooth muscle (types I and II), acted preferentially on *in vitro*-introduced phosphate in MAP2, with a very weak reactivity towards *in vivo*-incorporated ^{32}P (Murthy and Flavin, 1983; Murthy et al., 1985). Whether phosphate groups in NF proteins possess a similar property is not known.

The electrophoretic mobility of NF proteins was changed upon phosphatase treatment. As with that observed in bovine NF (Julien and Mushynski, 1982; Carden et al., 1985; Kaspi and Mushynski, 1985; and the present paper), we found that, in human NF, the increase of mobility was more prominent in NF-H than in 2 other proteins, reflecting the extent of NF dephosphorylation. It was already recognized (Kaufmann et al., 1984) that the molecular weight of NF proteins, as determined by the SDS-gel electrophoretic analysis, was much higher than that determined by other techniques. Now it becomes more evident that the discrepancy is due to a large extent to heavy phosphorylation of NF proteins (Carden et al., 1985; Georges et al., 1986; and the present paper). It remains to be tested whether a complete dephosphorylated NF could be shown, using SDS-gel electrophoresis, with a molecular weight comparable to that found by Kaufmann et al. (1984).

The location of the phosphate groups that reacted with phosphatase of the NF molecule remains unknown. A lack of, or weak, reactivity of 2 monoclonal antibodies, known to recognize phosphorylated sites on NF side arms (Ksiezak-Reding and Yen, 1987), with dephosphorylated NF shows that at least some phosphatase-reactive sites are located on the middle portion of the

projection arm of NF-H and NF-M. The other areas of the molecule could be suggested as locations of other, less reactive, groups, e.g., as places limited by the spatial orientation of the molecules in a filament. NF-L, whose phosphate groups were not removed by phosphatase, is believed to form a backbone of the filament, or to be located more centrally than the other 2 NF polypeptides (Willard and Simon, 1981; Chin et al., 1983). In this respect, our data did not support the hypothesis of equal (Francois et al., 1984) exposure of the NF polypeptides to the outside of the filament structure. A detailed localization of phosphate groups on the NF polypeptides is necessary for further discussion.

The carbocyanine dye-binding studies showed that the stain is useful for detecting phosphorylated cytoskeletal proteins, including NF, tubulin, MAP1, MAP2, and tau. According to our analysis of dye binding to the NF triplet, the amount of the blue complex formed is proportional to the protein content and each of the polypeptides has its own characteristic constant. The precise mechanism involved in the staining of phosphoprotein is not known, although it depends on the presence of anionic centers on the protein molecule. Carbocyanine dye has been shown to interact with the phosphoproteins such as casein or phosvitin (Green et al., 1973), as well as with other macromolecules containing sialic acid (Green and Pastewka, 1975; King and Morrison, 1976) or clusters of acidic amino acids as in Ca^{2+} -binding proteins (Campbell et al., 1983; Caday and Steiner, 1985). Since the removal (or blockage by a ligand) of these anionic sites leads to a loss of the blue complex, the carbocyanine dye has been used as a structural probe to monitor ligand-dependent conformational changes in calmodulin (Caday and Steiner, 1985).

The possibility that the sialic acid residues, rather than phosphate groups in NF, could be responsible for the formation of the blue complex with "Stains-all" was ruled out by the lack of effect of neuraminidase on either the electrophoretic mobility or staining characteristic of any of the NF triplet proteins (unpublished observations).

The intensity of staining of the NF polypeptides by carbocyanine dye did not always correlate with the phosphate content of the proteins, especially in NF-H. The relative unit of dye binding/mol protein for NF-H was similar to that for NF-M, and it was only 2–3 times that of NF-L. Treatment of NF proteins with alkaline phosphatase affected the binding of the dye to NF-H far more than to NF-M. The dephosphorylated NF-H, even though retaining nearly half of its phosphate groups, was no longer detected by the dye, suggesting that the 2 pools of phosphate groups differ substantially, perhaps in their location and/or their influence on the shape of the NF-H molecule, in analogy with the anionic centers in calmodulin (Caday and Steiner, 1985). All the results suggest that the arrangement or the distribution of phosphate groups in NF proteins can be categorized into at least 2 groups; one is easily removed by the phosphatase and the other is not. In NF-H and NF-M the phosphatase-reactive sites are involved in formation of blue complex with "Stains-all," and they exclusively bind 2 monoclonal antibodies. Whether the presence of these different phosphorylated sites depends on the action of different protein kinases or/and phosphatases requires further investigation. It is possible that some phosphate groups in the NF triplet proteins could be incorporated by Ca^{2+} -calmodulin-dependent protein kinase (Valiano et al., 1985) and others by a type II cyclic AMP-dependent protein kinase (Leterrier et al., 1981) associated with MAP2

(Theurkauf and Vallee, 1982). The precise location of these different phosphorylation sites and their role in the pathology of NF proteins require further study.

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