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Identification of the major multiphosphorylation site in mammalian neurofilaments

(phosphorylation/microtubule-associated protein/neurofibrillary tangles)

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ABSTRACT **The sequence Lys-Ser-Pro-Val-Pro-Lys-Ser-Pro-Val-Glu-Glu-Lys-Gly repeats six times serially in the human midsized neurofilament (NF) protein (NF-M). To establish whether Lys-Ser-Pro-Val(Ala) is the major site for *in vivo* NF phosphorylation, peptides based on the human NF-M repeat were synthesized and chemically phosphorylated. These synthetic peptides were probed with 515 monoclonal antibodies (mAbs) that were raised to, and distinguished, several differentially phosphorylated forms of NF proteins. Studies with 95 of those mAbs that recognized the peptides before and after chemical phosphorylation demonstrated that a highly immunogenic epitope shared by the peptides is present in NFs from all species tested, including invertebrates. This suggests the phylogenetic conservation of a major NF phosphorylation site. Lastly, a cross-reactive antigenic determinant shared by the peptides and the major NF phosphorylation site was shown to exist in neurofibrillary tangles of patients with Alzheimer disease as well as in two neuron-specific microtubule-associated proteins (MAPs)—i.e., MAP2 and tau.**

Neurofilaments (NF) are the most abundant cytoskeletal structures in large-diameter axons, where they provide mechanical support and appear to regulate fiber caliber and, possibly, the related property of nerve conduction velocity (1). NF proteins are assembled into 10-nm-diameter filaments via coiled-coil interactions between structurally conserved "rod" regions (310 amino acids) that are present in the high, medium, and low M_r (NF-H, NF-M, and NF-L, respectively) NF subunits (2–4). However, NF-M and NF-H have long sequence extensions (400–600 amino acids) on the COOH-terminal side of their homologous rod regions (2, 3, 5), and it is these additional domains that project from the NF backbone (6). Thus, the projecting domains of NF-M and NF-H probably determine the surface properties of NFs, and it is precisely these domains that are extensively phosphorylated *in vivo* (up to 50 mol of phosphate per mol of protein) (7, 8). Accordingly, phosphorylation may actively modulate the surface properties and, possibly, the functional interactions of NFs. Recently, the gene for human NF-M was cloned (9), and the predicted protein sequence reveals an unusual repeat near the middle of its COOH-terminal half—i.e., the projecting domain. Within this segment, the sequence Lys-Ser-Pro-Val-Pro-Lys-Ser-Pro-Val-Glu-Glu-Lys-Gly is repeated serially 6 times to yield a 78-amino acid unit in which Lys-Ser-Pro-Val occurs 12 times. Moreover, a highly related sequence (Lys-Ser-Pro-Ala) is tandemly repeated a large, but as yet unspecified, number of times in the COOH-terminal projecting domain of the more heavily phosphorylated rat NF-H (10). If such sequences represent recognition sites for NF-directed kinase(s), then the addition of multiple phosphate groups to NF-M and NF-H *in vivo* may result from the repetitive phosphorylation of a restricted phosphate acceptor sequence.

The aim of this study was to investigate the hypothesis that the serial repeat described above is a multiphosphorylation site in NF.

MATERIALS AND METHODS

Synthetic Peptides. Peptides corresponding to the 13-amino acid repeat sequence Lys-Ser-Pro-Val-Pro-Lys-Ser-Pro-Val-Glu-Glu-Lys-Gly (designated 1–13nP) in human NF-M (residues 614–626) or a trimer (designated 1–39nP) thereof (residues 614–652) were synthesized on solid phase using a PAM resin and Boc amino acid pentafluorophenyl esters as coupling reagents (11). Phosphopeptides were then generated by chemically phosphorylating (12) the serines in these peptides (termed 1–13P and 1–39P, respectively). We then confirmed that all serine residues in both 1–13P and 1–39P had covalently linked phosphate (13).

Antibodies. All monoclonal antibodies (mAbs) used in this study were previously generated to NF-H and NF-M from diverse mammalian species (cow, human, and rat) (8, 14–16). These mAbs were specific for either NF-M or NF-H or recognized both NF-M and NF-H in their phosphorylated or nonphosphorylated forms when tested with human NF preparations. The mAbs generally were used as supernatants, but in selected cases, mAbs in ascites were also used. Rabbit anti-peptide antibodies were prepared by immunizing rabbits with either 1–39nP or 1–39P peptides (300 μg of peptide per rabbit per immunization) as described for the immunization of rodents (14–16).

Preparation of NFs and Microtubule-Associated Proteins (MAPs). NFs were prepared from spinal cords of bovine, rat, and human as reported (8, 14, 15). Enriched NF preparations from other species (i.e., mouse, hamster, rabbit, chicken, duck, *Xenopus*, squid, lamprey, and *Myxicola*) were obtained from freshly isolated spinal cords by direct solubilization of tissue in boiling Laemmli sample buffer at a concentration of 10 mg/ml (wet weight). Bovine MAPs—i.e., MAP2 and tau—were isolated as described (17).

Immunochemical and Immunocytochemical Methods. ELISA was conducted as described (15) except that 50 μl of each of the synthetic peptides (at 10 μg/ml) was dried onto 96-well ELISA plates and used as antigen to be tested for antibody recognition.

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Abbreviations: AD, Alzheimer disease; MAP, microtubule-associated protein; mAb, monoclonal antibody; NF, neurofilament; NF-H and NF-M, high and medium M_r NF subunits, respectively; NFT, neurofibrillary tangle.

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For immunoblotting experiments, NF-enriched preparations, MAP2, and tau were separated on 7.5% $NaDodSO_4$/polyacrylamide gel, transferred onto nitrocellulose replicas, and immunoblotted with various mAbs as described in detail elsewhere (15). For the immunodot assay, synthetic peptides (0.01–1 μg) were spotted onto nitrocellulose replicas (0.22 μm), air-dried, and processed like the immunoblots.

Immunohistochemical studies were conducted on Bouin's fixed, paraffin-embedded tissue sections cut from hippocampi of Alzheimer disease (AD) brains. These studies were performed exactly as recently described (15).

Dephosphorylation. Dephosphorylation of NF proteins on nitrocellulose replicas and of tissue sections with *Escherichia coli* alkaline phosphatase (type III-N) was also conducted as described (refs. 8 and 14, respectively).

RESULTS AND DISCUSSION

Many mAbs Raised to Authentic NFs Recognize Phosphorylated and Nonphosphorylated Forms of the 13-Amino Acid Repeat. Our strategy for analyzing the major phosphorylation site(s) of NF-M and NF-H utilized mAbs to native NF subunits. To this end, all four synthetic peptides—i.e., 1–13nP, 1–39nP, 1–13P, and 1–39P—were tested for recognition with a total of 515 mAbs from our library of mAbs previously generated to NF-H and NF-M from rat, cow, and human tissue sources. These mAbs are capable of distinguishing NF-H and NF-M in their phosphorylated and nonphosphorylated forms. Table 1 summarizes the ELISA data that confirmed the results of the immunodot studies. Furthermore, ≈19% (95/515) of the mAbs recognized at least one of the test peptides, showing that the human NF-M repeat is highly immunogenic and that an identical or related sequence also exists in NF-M and NF-H from many different mammalian species (Table 2).

The immunochemical data allow separation of our mAbs into six categories (Table 1). Types A and B mAbs are specific only for determinants present in the synthetic phosphopeptides—i.e., either 1–39P alone (type A) or both 1–13P and 1–39P (type B). Both types A and B mAbs are specific for phosphorylated forms of intact NF-M and/or NF-H as determined by immunoblot analysis (8, 14–16). That a great majority (64/95) of the available mAbs specific for the synthetic peptides are either type A or B is not surprising, since most of our mAbs (496/515) resulted from immunization with NF protein extracts in which the highly phosphorylated variants of these proteins predominate (8, 14–16). This is particularly true for human NF-M since 34/43 of mAbs raised to human NF immunogens recognized both 1–13P and 1–39P peptides (Table 2). This provides additional evidence that the human NF-M repeat is an immunodominant region since it elicits the major antigenic response by the immune systems of rats immunized with this subunit.

Types C and D mAbs recognized only nonphosphorylated peptides—i.e., 1–13nP and/or 1–39nP (Table 1). Both of these types are specific for nonphosphorylated or enzymatically dephosphorylated forms of intact NF-M and/or NF-H subunits. Of these 14 mAbs, 9 were produced in response to immunization with enzymatically dephosphorylated authentic NF (Table 2). However, 5 resulted from immunization with native rat NF-M (the substantially phosphorylated variant of this subunit). This suggests that the gel-purified rat immunogen (15) was contaminated with nonphosphorylated or poorly phosphorylated NF-H. This is understandable since nonphosphorylated forms of rat NF-H migrate in $Na\text{-}DodSO_4$/polyacrylamide gels to a position extremely close to the phosphorylated forms of rat NF-M (15). Thus, with respect to the phosphorylation state of the synthetic peptides, types C and D mAbs are probes complementary to types A and B mAbs. However, it does not necessarily follow that all 4 types of mAbs recognize the exact same sites on the respective peptides.

Table 1. mAb recognition of different synthetic peptides

Peptide					
1–39P	1–13P	1–39nP	1–13nP	Type	No. of mAbs
+	−	−	−	A	56
+	+	−	−	B	8
−	−	+	−	C	11
−	−	+	+	D	3
+	−	+	−	E	6
+	+	+	+	F	12

The ability of mAbs to bind to the different peptides was tested by ELISA using 50 μl of each peptide (at 10 μg/ml) dried onto 96-well ELISA plates and spent mAb supernatants.

Types E and F mAbs comprise a distinctly different third set of probes since they recognized the synthetic peptides independent of their phosphorylation state (Table 1). Type E mAbs were shown to bind both 1–39nP and 1–39P peptides, while type F mAbs recognized all 4 synthetic peptides (i.e., 1–13nP, 1–13P, 1–39nP, and 1–39P). Correspondingly, both types E and F mAbs recognized epitopes that also are independent of the phosphorylation state of intact NF-M and/or NF-H.

In ELISA and/or immunodot assays, the titration curves produced with mAbs from each of these 6 distinct types of mAbs revealed that all 6 types had consistently higher affinities for the triple repeats than for the corresponding single unit peptides regardless of whether the peptides were phosphorylated (data not shown).

Antisera generated in rabbits immunized with the 1–39nP and 1–39P peptides were tested in immunoblots using human NF preparations. Both of these antisera were shown to bind to NF-M and NF-H (data not shown). However, the antisera raised to the 1–39P peptide recognized phosphorylated NF subunits better than the antisera raised to the 1–39nP peptide, while the latter antisera detected enzymatically dephosphorylated NF-M and NF-H better than the 1–39P antisera. These results confirm that this repeat sequence is derived from NF-M, and they also raise the possibility that NF-H may have a similar repeat domain. The cross-reaction of these antisera with both NF-H and NF-M before and after enzymatic dephosphorylation probably reflects the presence of antibodies in the antisera that see phosphorylation-independent epitopes similar to the class E and F mAbs described above.

Reactivities of Peptide Specific mAbs with NF Subunits of Six Different Mammalian Species. All 95 peptide-specific mAbs were tested by the immunoblot technique to probe electrophoretically separated NF subunits from human, rat, mouse, bovine, rabbit, and hamster sources in identical nitrocellulose replicas (Table 3). This permitted us to correlate the NF subunit domain location and phosphorylation state of the epitopes recognized by these mAbs with the ability of these mAbs to recognize phosphorylated versus nonphosphorylated synthetic peptides.

Type A mAbs. All but 3 of the 56 type A mAbs recognized both phosphorylated NF-M and NF-H from all 6 species (Table 3). However, in humans, the immunoreactivity of these mAbs for NF-M was greater than for NF-H, while the reverse was true for all other species tested here. The three other mAbs detected human NF-M, but they exhibited variable recognition of NF-M from other species (Table 3).

Our results indicate that this immunodominant multiphosphorylated repeat domain from human NF-M elicited a large number of mAbs when native human NF-M was used as the immunogen. Furthermore, since type A mAbs only recognize 1–39P peptide, but not 1–13P peptide, our data suggest that the secondary structure of this repeat domain, rather

Table 2. Distribution of peptide-specific mAbs from different panels of mAbs

Source of NF immunogen	Peptide-specific mAbs/total tested	Number of each type mAb					
		A	B	C	D	E	F
Human NF-M (HO)	35/43	32	2	0	0	0	1
Rat NF-M (RMO)	28/295	6	6	5	0	6	5
Bovine NF-M(Oc)	6/99	6	0	0	0	0	0
Bovine NF-H (Ta)	14/60	12	0	0	0	0	2
Rat NF-M, dp (RMdO)	9/11	0	0	5	2	0	2
Rat NF-H, dp (RMdT)	1/1	0	0	0	1	0	0
Bovine NF-M/H, dp (dP)	2/6	0	0	1	0	0	1

The mAbs used here were generated as described (8, 14–16). dp, Dephosphorylated; HO, Oc, Ta, and dP mAbs are rat–mouse hybridomas; RMO, RMdO, and RMdT are mouse–mouse hybridomas. The mAbs were tested for their peptide specificities by the ELISA assay.

than the primary sequence, is responsible for the observed high degree of immunogenicity.

It is interesting to note that some type A mAbs can be obtained by using rat NF-M, bovine NF-M, and bovine NF-H as immunogens. Complete sequence data on rat NF-M do not reveal a similar 1–13nP amino acid repeat in tandem (18). However, two peptides, each of which contains the sequence Lys-Ser-Pro-Val repeated twice as in the 1–13nP peptide, have been found in two different locations of the COOH-terminal domain of rat NF-M (18). At present we do not know the exact sequence of bovine NF-M or NF-H. However, our previous studies have shown that bovine NF-M is immunologically very different from NF-M of a wide variety of other mammalian species (16), while immunological properties of bovine NF-H appear to be conserved or well represented across a number of mammalian species. Since the type A mAbs recognize the 1–39P peptide in addition to phosphorylated isoforms of NF-M and NF-H from these diverse mammalian species, they may be regarded as mAbs with a broad yet sharply circumscribed specificity for a group of closely related epitopes. An attractive interpretation of this observation is that a large number of different secondary structures are possible due to the highly charged and peripheral location of these phosphorylated regions in NF-M and NF-H regardless of the presence of a consecutive repeat of 1–13 amino acids as in human NF-M.

Type B mAbs. Six of these mAbs recognized NF-M only, while two others detected both NF-M and NF-H. At present, we do not know whether type B mAbs recognize a sequence within the 1–13P peptide or the secondary structure of this peptide. However, these mAbs do not recognize two other synthetic phosphopeptides with the sequences Lys-Ser-Pro-Val-Pro-Ser-Ser-Pro-Cys-Glu-Glu and Lys-Ser-Pro-Ala-Glu, suggesting that none of these mAbs recognizes the phosphorylated site Lys-Ser-Pro-Val(Ala).

Type C mAbs. These mAbs were found to bind to both dephosphorylated human NF-M and NF-H, but in other species they primarily detected dephosphorylated NF-H. Since these mAbs appear to recognize conformational epitopes that are determined by the secondary structure of the repeat domain, these data suggest that human NF-M is the only form of mammalian NF-M that has a multiphosphorylation site consisting of a repeat region among the six mammalian species tested. In contrast, all of the mammalian species tested here probably have a repeat domain located in NF-H rather than NF-M. Thus, in species other than humans, NF-H rather than NF-M may contain the major multiphosphorylation site of the NF triplet proteins.

This interpretation is strengthened further by the data showing that the type C mAbs bind almost exclusively to rat NF-H, even though rat NF-M and NF-H do not have a repeat domain with the same amino acid sequence as the 1–39nP sequence from human NF-M (9, 10, 18). However, rat NF-H does contain a repeat domain with the sequence Lys-Ser-Pro-Ala-Glu-Ala (10) repeated at least four times. Thus, homologies of secondary structure among these domains may account for the observation that type C mAbs bind to both NF-H and NF-M in many nonhuman mammalian species.

Table 3. Reactivities of peptide-specific mAbs with different species

Type	No. of mAbs	Human		Rat		Mouse		Bovine		Rabbit		Hamster	
		H	M	H	M	H	M	H	M	H	M	H	M
A	53	53	53	53	53	53	53	53	53	53	53	53	53
	3	0	3	0	2	0	1	0	0	0	2	0	2
B	2	2	2	2	2	2	2	2	2	2	2	2	2
	6	0	6	0	6	0	6	0	6	0	6	0	6
C	5	5	5	5	0	5	0	5	0	5	5	5	0
	4	4	4	4	0	4	0	4	0	4	0	4	0
	2	2	2	2	2	2	2	2	0	2	2	2	2
D	3	3	3	3	3	3	3	3	0	3	3	3	3
E	6	0	6	0	6	0	6	0	6	0	6	0	6
F	4	4	4	4	4	4	4	4	4	4	4	4	4
	3	0	3	0	3	0	3	0	3	0	3	0	3
	2	0	2	0	2	0	2	0	0	0	0	0	2
	1	1	1	1	1	1	0	1	0	1	1	1	0
	1	0	1	0	1	0	1	0	0	0	1	1	1

Peptide-specific mAbs were tested for reactivity with NF subunits from six different mammalian species by using the immunoblot technique (15). Freshly isolated spinal cords from each species were solubilized directly using Laemmli sample buffer at 10 mg/ml (wet weight). NF proteins were separated by 7.5% $NaDodSO_4$/PAGE and transferred to nitrocellulose paper as described (15). The mAb reactivities were detected by the peroxidase-antiperoxidase technique. The total number of mAbs of each type is listed, and these mAbs are further distinguished by their ability to recognize the NF-H and/or NF-M subunits in the different species listed.

Type D mAbs. These mAbs detected NF-M and NF-H in all species examined except bovine. To evaluate the ability of these mAbs to recognize sequences similar, but not identical, to those in the peptides that model portions of authentic human NF-M, we tested type D mAbs with two other synthetic peptides with the sequence Lys-Ser-Pro-Val-Pro-Ser-Ser-Pro-Cys-Glu-Glu and Lys-Ser-Pro-Ala-Glu. Two of the three mAbs in this category bind strongly to these two peptides. Since the common amino acid sequence between the 1–13nP peptide and these two other peptides is the sequence Lys-Ser-Pro-Val(Ala), we conclude that the recognition site for these two mAbs is Lys-Ser-Pro-Val(Ala). It is interesting to note that the phosphorylation site characterized by Lys-Ser-Pro-Val(Ala) is present in NF-M and NF-H of all species tested except bovine NF-M, which is immunologically different from NF-M of other species. We have therefore confirmed our previous results and have extended the observation that bovine NF-M does not contain the sequence Lys-Ser-Pro-Val(Ala) as a phosphorylation site. However, since many of the other mAbs (particularly type A mAbs) bind to bovine NF-M, this suggests that, although the sequence of the phosphorylation site may be different, many of the secondary structure characteristics of the phosphorylation site appear to be conserved among mammalian NFs.

Type E mAbs. Type E mAbs were specific only for NF-M in all six species tested. It is likely that both 1–39nP and 1–39P peptides share some secondary structural characteristics that, interestingly, are found in mammalian NF-M but not NF-H.

Type F mAbs. The 11 type F mAbs exhibited the greatest variation in specificity for mammalian NF subunits. We do not know yet whether these mAbs recognize a sequential or a conformational determinant. However, none of the type F mAbs recognized either the phosphorylated or the nonphosphorylated form of the sequence Lys-Ser-Pro-Val(Ala).

Immunohistochemical Staining Pattern of Peptide-Specific mAbs in Nervous System Tissue. Previously, we established that there is a predictable correlation between the biochemically distinct NF-H and NF-M isoforms, as detected by our mAbs and the staining patterns produced by these same mAbs in nervous system tissue (15). Five different classes of NF-H and NF-M isoforms were defined by five classes of mAbs as follows: (*i*) P[−] mAbs recognized only extensively dephosphorylated NF-H, or both NF-H and NF-M, and these mAbs preferentially stained neuronal perikarya and dendrites; (*ii*) P[+] mAbs recognized only phosphorylated forms of NF-H and/or NF-M, and these mAbs stained axons more strongly than perikarya; (*iii*) P[++] mAbs stained axons almost to the exclusion of perikarya, and they recognized forms of NF-H and NF-M that were more phosphorylated than those detected by P[+] mAbs; (*iv*) P[+++] mAbs stained only axons, and they recognized only the most extensively phosphorylated forms of NF-H and NF-M; (*v*) P[I] mAbs recognized epitopes that were independent of the phosphorylation state of these subunits, and many of these mAbs stained paraffin-embedded tissue sections poorly, although a few of these mAbs stained tissue sections in a manner similar to that observed with the P[+]-type mAbs.

We reexamined the staining patterns produced by these peptide-specific mAbs by using sections of human and rat cerebellum and spinal cord to correlate the staining patterns with the peptide specificities of these mAbs and the NF-H and NF-M isoforms they recognized. Our results showed that: (*i*) types A and B mAbs, which recognized the phosphorylated peptides, produced a staining pattern similar to that of P[+]-type mAbs; (*ii*) types C and D mAbs, which recognized only the nonphosphorylated peptides, produced staining patterns similar to those obtained with the P[−] mAbs; (*iii*) types E and F mAbs, which recognized both phosphorylated and nonphosphorylated peptides, stained tissue sections weakly, like the P[I] mAbs. The latter observation suggests that [under the conditions we have used to process and probe these tissues with our mAbs (15)] the antigenic determinants recognized by the types E and F mAbs are probably inaccessible to the mAbs because of their location within the interstices of the filament or as a result of distortions in molecular conformation that are induced by chemical fixation.

From these data we conclude that the phosphorylation of the NF-M and NF-H multiphosphorylation site gives rise to the P[+] isoforms of these subunits and that the phosphorylation sites for the P[++] and P[+++] isoforms of NF-M and NF-H are unknown and remain to be determined.

Immunoreactivity of Peptide Specific mAbs with Other Submammalian Species. Submammalian species may have a repeat domain similar to that found in mammalian NFs as the major phosphorylation site. Thus, NF preparations from several submammalian species were examined by the immunoblot method to establish this possibility. This was accomplished by using NF extracts from avian species (chicken, duck), an amphibian (*Xenopus*), a mollusc (squid), a cartilaginous fish (lamprey), and an annelid (*Myxicola*). We noted that at least five peptide-specific mAbs recognized one or more NF subunits in each of the submammalian species tested. Some of these species (chicken, duck, *Xenopus*) have three NF subunits (like mammals), while others (e.g., lamprey) have only one (19). Several peptide-specific mAbs, particularly the types A and C mAbs, produced immunobands that corresponded to NF-M alone in chicken, duck, and *Xenopus*. Although the immunobands produced with the NF preparations from squid and *Myxicola* were highly variable, selected types A and C mAbs yielded immunobands in these two species that corresponded with mammalian NF-H. Finally, in NF extracts from lamprey, which has just one NF subunit of M_r 180,000 (19), only the type E mAbs yielded immunobands that corresponded to this subunit. These data suggest that a repeat domain, immunologically similar to the major multiphosphorylation site in mammalian NFs, may exist in submammalian NFs.

Immunoreactivities of Peptide-Specific mAbs with Other Cytoskeletal Proteins. To evaluate the possibility that the NF multiphosphorylation site may exist in non-NF cytoskeletal proteins, we used our peptide-specific mAbs to detect determinants related to this site in other cytoskeletal proteins, such as the high and low M_r MAPs, and we found that several of the type A-specific mAbs cross-reacted with MAP2 and tau. Thus, a repeat domain similar to the multiphosphorylation site in NF-H and NF-M may exist in MAP2 and tau. Finally, the two type D-specific mAbs, which recognize the sequence Lys-Ser-Pro-Val(Ala), cross-reacted only with tau. It is known already that tau proteins share antigenic determinants with NF-M and NF-H (20, 21). Available sequence information on tau proteins suggests that Lys-Ser-Pro-Val is indeed present in this polypeptide (K. Kosik, personal communication).

Identification of an Abnormal Phosphorylation Site in AD Tangles. Recent reports indicate that an abnormally phosphorylated form of tau is the major antigenic component of neurofibrillary tangles (NFTs) in AD (22). Since tau and NF proteins share immunological similarities, studies were undertaken to determine whether any abnormal tau phosphorylation sites in NFTs include the sequence Lys-Ser-Pro-Val. Sections of hippocampus from two AD patients were immunostained with the type D mAbs, which bind to the sequence Lys-Ser-Pro-Val(Ala). Both mAbs immunostained normal neuronal perikarya, dendrites, and axons, while NFTs were negative or weakly stained (Fig. 1*A*). However, after adjacent sections were subjected to enzymatic dephosphorylation (14), many intensely stained NFTs were observed (Fig. 1*B*). These results suggest that Lys-Ser-Pro-Val repre-

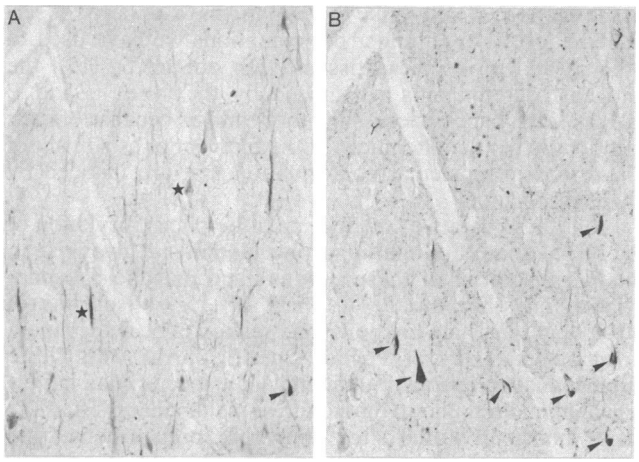

FIG. 1. Immunostaining of AD NFTs in adjacent sections of hippocampus with a type D mAb (RMdO 19) before (*A*) and after (*B*) enzymatic dephosphorylation of the section with alkaline phosphatase. Note the branching blood vessels in both *A* and *B*. More RMdO 19-stained NFTs (arrowheads) are present in *B* than in the same area in *A*. The stars in *A* identify an immunostained dendrite and tangle-free neuron. (×90.)

sents at least one of the abnormal phosphorylation sites in AD NFTs.

Conclusions and Speculations Concerning Multiphosphorylation Sites in NF Polypeptides and Other Cytoskeletal Proteins of Mammalian and Submammalian Species. We have identified the major multiphosphorylation site of NF proteins and have shown that it consists of Lys-Ser-Pro-Val repeats that are present in NF-M and NF-H of diverse mammalian species and in the NFs of many submammalian species. Thus, this site may be common to NFs of all, or nearly all, species and it may support unique functions within the axon. The observation that NFs from species such as *Xenopus* and squid are recognized by peptide-specific mAbs suggests the early evolution of NFs from the IF gene family. Furthermore, this multiphosphorylation site is also the immunodominant region of NFs. The latter conclusion is not based solely on our library of mAbs, since many commercially available anti-NF mAbs bind to our synthetic peptides (data not shown). For example, among the Sternberger–Meyer mAbs (23) tested, SMI-31 is a type A, SMI-32 is a type C, and SMI-33 is a type D mAb. Our observation that several of the peptide-specific mAbs cross-react with MAP2 and tau, together with reports by others (20, 21) that some mAbs raised to NF proteins also cross-react with MAP2 and tau, suggests the existence of structural and/or sequence homologies between these two groups of cytoskeletal proteins. The significance of phosphorylation in regulating the function of these two different groups of cytoskeletal proteins in the neuron remains to be determined.

At the present time, we do not know the structural changes that are wrought by the phosphorylation of intact NFs. Nevertheless, the phosphorylation of a repeat domain, such as that described here, will introduce a large number of negative charges into the COOH termini of NF-M and NF-H, and this could induce a repositioning or extension of NF-H and NF-M sidearms. If this indeed is the case, we may have identified the region of NF-H and NF-M that regulates this movement as a consequence of altered electrostatic charges that are in turn mediated by the phosphorylation of this region. Accordingly, the synthetic peptides corresponding to the repeat domain that we describe here will be useful for identifying the kinases and/or phosphatases that regulate the phosphorylation state of this domain. Furthermore, the peptide-specific mAbs used in this study represent molecular probes that can be exploited to monitor normal and pathological states of NF protein phosphorylation. This should permit the elucidation of the role of phosphorylation in NF function and also an understanding of the significance of aberrant protein phosphorylation in disease states such as AD.

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