Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ

(chemoarchitectonics/electroblot/immunocytochemistry/monoclonal peroxidase-antiperoxidase/neuronal individuality)

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ABSTRACT  The immunocytochemical staining patterns of 37 neuron-specific monoclonal antibodies previously described fell into four groups: (i) anti-synapse-associated, (ii) anti-neurofilibrillar, (iii) anti-perikaryonal-neurofilibrillar, and (iv) a single antibody reactive with a widely distributed epitope that covered the patterns of groups ii and iii. Antibodies of groups ii, iiii, and iv were shown to be specific to neurofilament triplet subunits, even though there was little overlap in staining patterns between groups ii and iii. We examined nine of these antibodies as to their ability to distinguish functional states of neurofilaments dependent upon phosphorylation. Upon digestion with phosphatase, electroblot staining of neurofilament components was abolished with the five antibodies from group ii, enhanced with the three antibodies from group iii, and unaffected with antibody iv. Immunocytochemical staining of Bouin-fixed paraffin sections of rat brain was unaffected by phosphatase pretreatment. With antibodies of group ii, digestion with trypsin also left staining unaffected, but when followed by digestion with phosphatase, staining was diminished with three out of five antibodies. In contrast, digestion with trypsin ablished all staining with each antibody from group iii. If followed by digestion with phosphatase, staining reappeared, but the group iii pattern was replaced by a group ii pattern. Staining of this pattern was again abolished upon a second treatment with trypsin. The antibody from group iv lost most of its groups ii and iii staining patterns when sections were digested with trypsin. The group ii pattern reappeared and, indeed, was enhanced upon a subsequent phosphatase treatment and was reduced again upon a second trypsin treatment. Staining by four out of five antibodies from group ii was inhibited by inorganic phosphate. The data indicate that certain nerve cell bodies, their dendrites, and at least proximal axons possess nonphosphorylated neurofilaments and that long fibers, including terminal axons, possess phosphorylated neurofilaments. We propose that phosphorylation may be a factor in stabilizing compacted forms of neurofilaments and that heterogeneity of the compacted structures may play a role in a possible multiplicity of function within individual nerve cells.
MATERIALS AND METHODS

Cytoskeletal preparations from rat brain stems obtained by the method of Chiu et al. (3) and Eng et al. (4) were applied in amounts ranging from 2 to 10 μg on gels containing 8% acrylamide, 0.375 M Tris-HCl (pH 8.8), and 0.1% NaDodSO₄ and were electrophoresed and electroblotted along with molecular weight standards by the method of Towbin et al. (6) as detailed (8). One strip containing M, standards and one containing cytoskeletal preparations in each blot were stained by Coomassie blue, and the rest of the strips containing cytoskeletal preparations were incubated at 32°C for 2.5 hr either in 0.1 M Tris-HCl, pH 8.0/0.01 M phenylmethylsulfonyl fluoride containing 43 μg of calf intestinal alkaline phosphatase (type VII, Sigma) per ml or in the same solution devoid of phosphatase.

Sagittal paraffin sections of brains (7 μm thick) from Sprague-Dawley rats perfused with Bouin’s fixative under Nembutal anesthesia were incubated (i) with trypsin (GIBCO) at 37°C for 10 min (400 μg/ml) in 0.05 M Tris-HCl, pH 7.6/0.3 M sodium chloride/0.02 M calcium chloride; or (ii) with calf intestinal phosphatase at 32°C for 2.5 hr (130 μg/ml) or for 18 hr (400 μg/ml) in 0.1 M Tris-HCl, pH 8.0/0.01 M phenylmethylsulfonyl fluoride; or (iii) with both trypsin- and phosphatase-containing buffers sequentially; or (iv) in these buffers without enzymes.

The incubated electroblots and the paraffin sections were stained immunocytochemically (9, 10) with mouse peroxidase-anti-peroxidase prepared from monoclonal anti-peroxidase (Clono PAP) (2, 6, 11) diluted 1:100 to contain 4.3 μg of peroxidase and 11.6 μg of anti-peroxidase per ml. Ascites fluids containing the following nine monoclonal antibodies were used as primary antibodies: five anti-neurofibrillar antibodies (06-17, 03-44, 06-68, 04-7, and 07-5), three anti-perikaryonal-neurofibrillar antibodies (02-135, 06-32, and 06-53), and the broadly reacting antibody 02-40. Antibodies were diluted in 0.05 M Tris-HCl, pH 7.6/0.3 M sodium chloride/1% normal goat or rabbit serum (depending on the species of secondary antibodies (9, 10)), except in special cases where 0.15 M sodium potassium phosphate (pH 7.6) was substituted for 0.5 M Tris-HCl. For electroblots, antibodies were applied in dilutions of 1:2,000 for 1 hr. For the paraffin sections, in each experiment, a series of antibody dilutions were applied for 24 hr, ranging from 1:1,000 to 1:320,000.

RESULTS

Treatment of electroblots with phosphatase abolished the immunocytochemical staining with the five anti-neurofibrillar antibodies (06-17, 03-44, 06-68, 04-7, and 07-5) (Fig. 1). It enhanced the staining with the three anti-perikaryonal-neurofibrillar antibodies (02-135, 06-32, and 06-53). Staining with the broadly reacting antibody 02-40 did not appear to be significantly affected.

No effect was noted when paraffin sections were treated with phosphatase at 130 μg/ml for 2.5 hr. However, if digested by trypsin prior to phosphatase, there was a slight decrease of staining with the anti-neurofibrillar antibody 04-7. Tryptic digestion alone had no effect on the staining with any of the anti-neurofibrillar antibodies. Yet, when tryptic digestion was followed by phosphatase at 400 μg/ml for 18 hr, staining was inhibited to about equal extents with the anti-neurofibrillar an-
tibodies 06-17, 04-7, and 07-5 (Fig. 2). No effect was noted with antibodies 03-44 and 06-68.

An entirely different pattern was observed with the three antibodies of the anti-perikaryonal-neurofibrillar group. Here digestion with trypsin alone abolished all immunocytochemical staining (Fig. 3). If trypptic digestion was followed by phosphatase at 130 µg/ml for 2.5 hr, staining reappeared, but the perikaryonal pattern (including attached axons and dendrites) remained invisible and was replaced by staining indistinguishable from the neurofibrillar pattern (basket cell fibers, transverse fibers in the cerebral cortex, and long fiber tracts) (Figs. 3 and 4). These fibers were never stained by the anti-perikaryonal-neurofibrillar antibodies in the absence of trypsin and phosphatase pretreatments. If the trypsin and phosphatase-treated sections were treated with trypsin a second time, the newly emerged neurofibrillar type of staining again disappeared (Fig. 3). Buffer post-treatment had no effect.

When the broadly reacting antibody 02-40, which stained both structures represented by the anti-neurofibrillar and the anti-perikaryonal-neurofibrillar antibodies, was applied to trypsin-treated sections, all staining became diminished (Fig. 5). If trypsin incubation was followed by phosphatase, the perikaryonal-neurofibrillar pattern of staining remained weak, but the neurofibrillar pattern became intensified beyond that of sec-

![Fig. 3](image-url) Rat cerebellar cortex. Paraffin sections are stained with anti-perikaryonal-neurofibrillar antibody 02-135 diluted 1:2,000. BB, preincubation in buffers. TB, preincubation in trypsin-containing buffer followed by incubation in buffer. TP, preincubation with trypsin followed by incubation with phosphatase. TPT, preincubation with trypsin followed by incubation with phosphatase and again by incubation with trypsin. (×200.)

![Fig. 4](image-url) Rat fastigial nucleus. Paraffin sections were stained with antibody 06-32 diluted 1:2,000. BB, preincubation in buffers (perikaryonal-neurofibrillar pattern); TP, preincubations with trypsin followed by incubation with phosphatase (neurofibrillar pattern). Sections were entirely blank (not shown) when preincubated with trypsin only or with trypsin followed by incubation with phosphatase and again by incubation with trypsin. (×200.)
Neurobiology: Sternberger and Sternberger

FIG. 5. Rat cerebellar cortex. Paraffin sections were stained with the broadly reactive antibody 02-40 diluted 1:2,000. BB, preincubation in buffer; TB, preincubation in trypsin-containing buffer followed by incubation in buffer; TP, preincubation with trypsin followed by incubation with phosphatase; TPT, preincubation with trypsin followed by incubation with phosphatase and again by incubation with trypsin. (×200.)

FIG. 6. Rat cerebellar cortex. Paraffin sections were stained with anti-neurofibrillar antibody 07-5 diluted 1:40,000. A, antibody diluted in Tris buffer (pH 7.6); B, antibody diluted in phosphate buffer (pH 7.6). (×200.)

tions only incubated in buffers. If followed by a second trypsin incubation, most staining again disappeared.

When antibodies were diluted in sodium potassium phosphate instead of Tris-HCl, staining was inhibited with four of the five anti-neurofibrillar antibodies (06-17, 06-68, 04-7, and 07-5) (Fig. 6). Staining was unaffected with antibody 02-40 or any of the three antibodies from the anti-perikaryonal-neurofibrillar group.

DISCUSSION

The effect of phosphatase on the electroblots stained with the five anti-neurofibrillar antibodies and the inhibition by phosphate buffer of the staining of paraffin sections with four of the five anti-neurofibrillar antibodies suggest that these antibodies are specific for phosphorylated epitopes. Because neurofilaments are extensively phosphorylated (7) and because each anti-neurofibrillar antibody exhibits its own distinct immunocytochemical staining pattern on sections (1) and in electroblots (6), it is unlikely that each of the anti-neurofibrillar antibodies reacts with the same phosphorylated epitope.

The enhancement of staining of electroblots by the three anti-perikaryonal-neurofibrillar antibodies after incubation in phosphatase suggests two interpretations. Conceivably, the antibodies react with phosphorylated and nonphosphorylated epitopes, but better with the latter. Alternatively, the 200 M₀ doublet and the 150 M₀ band of cytoskeletal preparations con-
sist of mixtures of phosphorylated and nonphosphorylated epitopes; the antibodies react only with nonphosphorylated epitopes, and a greater amount of nonphosphorylated epitopes becomes available upon digestion. The data with trypsin digestion of paraffin sections favor the latter alternative. Phosphorylated neurofilaments have been resistant to tryptic digestion. Therefore, the abolition of all staining with anti-perikaryonal-neurofilibrillar antibodies by trypsin suggests that they react solely with nonphosphorylated epitopes.

The conversion of the anti-perikaryonal-neurofilibrillar staining pattern to the nonoverlapping anti-neurofilibrillar pattern revealed by the anti-perikaryonal-neurofilibrillar antibodies after trypsin and phosphatase digestion suggests that at least a fraction of the fibers stained by anti-neurofilibrillar antibodies is susceptible to digestion with phosphatase at 130 μg/ml for 2.5 hr. However, this fraction is insufficient to greatly reduce the total staining of sections with anti-neurofilibrillar antibodies. In addition, the appearance of reaction with antibodies staining solely nonphosphorylated epitopes in structures that normally are revealed only with antibodies to phosphorylated epitopes suggests that anti-perikaryonal-neurofilibrillar antibodies react with the dephosphorylated form of the same epitope as do the anti-neurofilibrillar antibodies or, alternatively, that dephosphorylation permits accessibility to epitopes reactive with anti-perikaryonal-neurofilibrillar antibodies. The latter possibility is preferred because each monoclonal antibody seems to recognize different epitopes in heterogeneous neurofilaments (6), and it is unlikely that we have in our library of monoclonal antibodies an anti-perikaryonal-neurofilibrillar antibody corresponding exactly to the dephosphorylated epitopes with which each of our antibodies to phosphorylated epitopes reacts. Furthermore, as we shall see below, the phosphorylated form of neurofilaments seems to be less accessible to enzymes than the nonphosphorylated form, again supporting the concept of masking of epitopes by phosphorylation.

The reabolishment of the neurofilibrillar staining by anti-perikaryonal-neurofilibrillar antibodies after a second treatment with trypsin suggests again that nonphosphorylated forms of neurofilaments, irrespective of their location, are more susceptible to trypsic digestion than the phosphorylated forms, which have been unaffacted by trypsin. Apparently, phosphorylation increases compactness and order in neurofilament structure. Further support to this conclusion was obtained by the ineffectiveness of phosphatase on intact phosphorylated neurofilaments in fixed tissue, unless the tissue had been pretreated with trypsin and unless, in addition, large amounts of phosphatase (400 μg/ml for 24 hr) were used during prolonged incubation.

The resistance to phosphatase digestion of epitopes reactive with antibodies 03-44 and 06-68 is probably explained by their extreme inaccessibility. The failure of inhibition of one anti-neurofilibrillar antibody by phosphate (03-44) may be due to an especially high affinity of this antibody to a phosphorylated epitope or, alternatively, to simultaneous specificity for two adjacent phosphate groups, thus making its reaction with neurofilaments practically irreversible (12).

The fact that phosphorylated neurofilaments, despite their apparent inaccessibility to trypsin or phosphatase are, nevertheless, reactive with anti-neurofilament antibodies, may be due to the following reasons: (i) the antibodies may react with different sites than the enzyme, and (ii) the ionized and more antigenic group of phosphate (13) may be on the outside of phosphorylated neurofilaments, readily accessible to the antibody binding site, whereas the esterified and less antigenic groups may be sufficiently buried to be inaccessible to phosphatase.

Antibody 02-40 does not seem to react with a phosphorylated epitope or an epitope unmasked by dephosphorylation. It may react with a nonphosphorylated, more constant, and universal region of neurofilaments not subject to extensive heterogeneity and readily accessible to destruction by trypsin. Sequential trypsin and phosphatase treatment enhances the neurofilibrillar staining component of antibody 02-40, apparently because of the existence of additional 02-40-reactive epitopes in buried forms that are unmasked by the enzymes. After this unmasking, the second trypsin treatment again diminishes the enhanced neurofilibrillar staining, suggesting further loss of the intact site. Any compactness contributed to neurofilaments by phosphorylation is not essential for the light microscopic neurofilibrillar appearance of neurofilbrils because neurofilbrils are easily discernible at high power in perikarya with anti-perikaryonal-neurofilibrillar monoclonal antibodies. It is more likely that phosphorylation provides a further compacting or ordering of neurofilament structure, which may involve either neurofilaments alone or a possible association with tubulin (14) or other neurite constituents. The possibility of significance of phosphorylation in the functional complexity of the brain is made likely by the heterogeneity of neurofilaments (6). A conceivable role of the ordered structure, which compacting of heterogeneous neurofilaments may permit, could be a gating effect for the selective release at given synaptic sites of individual neuro-regulators among groups of neuroregulators coexisting in a single cell (15–16).

Scharrer and Sinden (17) have introduced the term "chemoarchitectonics" in their description of the optic tectum, in which brain morphology was brought a step further by the use of histochemistry. The detection by immunocytochemistry of a post-translational modification, such as phosphorylation, is an example in which chemoarchitectonics—i.e., the demonstration of metabolic or allosteric differences—extends the definition of neural structures indistinguishable by morphological criteria alone.

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