Phosphorylation-dependent Monoclonal Tau Antibodies Do Not Reliably Report Phosphorylation by Extracellular Signal-regulated Kinase 2 at Specific Sites*

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Analysis of phosphorylation of tau, the microtubule-associated proteins hyperphosphorylated in Alzheimer’s disease, is often performed using phosphorylation-sensitive monoclonal antibodies thought to report the presence or absence of one or two specific phosphorylations (cognate sites). Using several such antibodies we found a much more complicated relationship between phosphorylation at specific sites, as monitored by two-dimensional phosphopeptide mapping, and antibody recognition of these sites. Multiple phosphorylation of tau in several stages by the brain extracellular signal-regulated kinase 2 isoform PK40 suggested that phosphorylation at cognate sites is sometimes necessary (but not sufficient) to induce a change of antibody reactivity and in some cases is not even necessary in the background of multiple phosphorylation at other sites. No single phosphorylation site was found to be responsible for any level of gel mobility shift associated with phosphorylation. Tau acquired its maximal gel mobility retardation and final immunochemical profile at substoichiometric phosphorylation of most sites. This suggests that many alternate phosphorylation patterns can produce the same conformational and immunochemical presentation on sodium dodecyl sulfate-gel electrophoresis. Although PK40* prefers some phosphorylation sites, most notably Ser235, followed by Ser202 and Thr205, the phosphorylation of multiple Ser/Thr-Pro sites is not highly sequential. Ser396 is one of the least preferred sites and seems to require prior phosphorylation at Ser404.

The microtubule-associated tau proteins, normally involved in organizing the neuronal cytoskeleton, are a subject of intense interest because of their involvement in the neurofibrillary degeneration of Alzheimer’s disease (AD) (1) (for a review, see Ref. 1). Tau proteins associated with paired helical filaments (PHF) (2–4) and a substantial fraction of the soluble tau proteins from AD brains (5, 6) exhibit abnormal gel electrophoretic mobilities and abnormal immunochemical properties with phosphorylation-sensitive antibodies, indicative of a pathological hyperphosphorylated state. PHF-tau proteins are incompetent at binding and stabilization of microtubules and are believed to be responsible for the cytoskeletal abnormalities leading to neuronal dysfunction in AD.

Because of a large number of potential phosphorylation sites, a detailed analysis of normal or abnormal phosphorylation patterns is rather difficult. Therefore, the contribution of individual phosphorylation sites to tau function or dysfunction has largely remained obscure. Phosphorylation-sensitive antibodies appear to be convenient tools to assess the phosphorylation state of tau. Phosphorylated Ser/Thr-Pro sites are abundant in many cytoskeletal proteins and are very antigenic. Therefore, most antibodies are sensitive to these phosphorylation sites. This has directed much attention to the role of proline-directed tau phosphorylation, although other phosphorylations are clearly also important (7).

Some widely used monoclonal antibodies (mAbs) seem to probe the phosphorylation state of one or at most two Ser-Pro or Thr-Pro sites, inviting the possibility that immunoassays could yield detailed (even quantitative) information about the phosphorylation state of ex vivo tau isoforms. The experimental support for such mAb properties stems from site-directed mutagenesis experiments; conversion of single Ser or Thr residues into Ala led to dramatic alterations in immunoreactivity, either before or after phosphorylation in vitro. Monoclonal antibodies believed to indicate the phosphorylation state of individual sites are PHF-1, Tau-1, and AT8 (8–10) and the neurofilament mAbs SMI31, 33, and 34, cross-reacting with tau due to shared Lys-Ser-Pro motifs (11, 12).

There are several potential complications in such mutagenesis analyses. Conclusions are based on the immunochemical behavior of wild type versus mutant protein in very similar phosphorylation states, i.e. either completely unphosphorylated or fully phosphorylated with any of the available kinases. This may reveal that phosphorylation at a single site is necessary for the immunochemical change, although it may not be sufficient. Different results might be obtained if the phosphorylation states of wild type and mutant were very dissimilar (e.g. seemingly contradictory results regarding the cognate phosphorylation sites of SMI31, depending on the tau kinase used in Refs. 11 and 12). Another concern is that any mutation of the tau protein might alter the properties of distant domains in an allosteric manner. In that case any change in immunochemical properties could not be ascribed to the phosphorylation site that was mutated.

To complement existing studies of the relationship between tau phosphorylation and recognition by antibodies we used a correlative approach. The longest recombinant human tau isoform htau40 was phosphorylated to increasing levels with activated forms of ERK2. 32P-Labeled tau populations in various states of phosphorylation were analyzed by quantitative two-dimensional phosphopeptide mapping and antibodies thought to report recognition of sites in cognate patterns.
relationship between Tau phosphorylation and phosphoepitopes.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Human Tau and of PK40erk2**—The longest human 441-amino acid tau isoform was expressed from the htau40 cDNA (gift of Dr. Goedert) in *Escherichia coli* and purified by heat treatment and Mono S fast protein liquid chromatography as described before (15). PK40erk2 was purified from bovine brain according to previously published procedures and modifications (13, 15).

**Tau Phosphorylation**—3 μg of recombinant htau40 protein was incubated for 4 h at 37 °C with increasing amounts of PK40erk2 ranging from 0.002 to 0.2 μg in 30 μl of 50 mM HEPES, pH 7.0, 2 mM Mg acetate, 1 mM dithiothreitol, 0.5 mM ATP containing 25 cpm/pmol [γ-32P]ATP. For preparative purposes, 15 μg of htau40 was phosphorylated with 0.2 μg of PK40erk2 under identical conditions. Reactions were terminated with 20 μl of 50 mM EDTA or, in preparation for SDS-PAGE, by boiling with 20 μl of sample buffer. For determination of phosphorylation stoichiometry, samples stopped with EDTA were applied to GFC glass filters wetted with 10% trichloroacetic acid, 2% sodium pyrophosphate. After extensive washing with trichloroacetic acid and sodium pyrophosphate, 32P incorporation was determined by liquid scintillation counting.

**SDS-PAGE and Western Blotting**—Aliquots of incubation mixtures stopped with sample buffer were separated on 10% SDS-PAGE (Novex) for Coomassie Blue R-250 staining or Western blotting. Gels were slightly overrun to provide for better separation of mobility isoforms of tau. Tau proteins were excised from the gel and prepared for tryptic digests as described below. For Western blotting, sample aliquots corresponding to 0.1–0.5 μg of tau were transferred overnight at 4 °C, 50 μl onto nitrocellulose (16). Blots were blocked with 5% dry milk in 10 mM phosphate-buffered saline, Triton X-100, pH 7.2 (dilution buffer) and incubated for 2–4 h with mAbs Tau-1, 1,500; PHF-1, 1:100; and SMI31, 33, and 34 at 1:1,000 each in 5% dry milk/dilution buffer. Blots were stained with alkaline phosphatase-coupled goat anti mouse polyclonal antibody (1,300) with a nitro blue tetrazolium staining kit (Life Technologies, Inc.).

**Two-dimensional Tryptic Phosphopeptide Mapping**—Tau protein bands excised from Coomassie Blue-stained SDS-PAGE were washed twice in 0.5 ml of 10% methanol and once in 0.5 ml of water and 50 mM NH₄HCO₃. Gel slices were minced with a forceps and incubated overnight at 37 °C with 10 μg of bovine trypsin (Sigma, tosylphenylalanin chloromethyl ketone-treated) in 100 μl of 50 mM NH₄HCO₃. The supernatant was removed, and gel slices were extracted twice for several hours with 200 μl each of 50 mM NH₄HCO₃. Extraction yield of 32P-peptides was monitored by Cerenkov counting of gel slices and was generally >95%. Combined slice extracts were concentrated by lyophilization to about 100 μl, and 5 μg of fresh trypsin was added for a repeated digest over 6 h. Digests were evaporated to dryness and lyophilized three times with each 200 μl of water. Residues were taken up in 50 μl of peptide electrophoresis buffer (formic acid/acetate buffer/water 22:78:900) and evaporated again. Peptides were dissolved in 2 μl of peptide electrophoresis buffer and applied to a 20 × 20-cm cellulose plate (Merck). Peptide maps were developed as described (17). The first dimension was peptide electrophoresis buffer at 1,000 V for 50 min. The second dimension was isobutyric acid/n-butyl alcohol/pyridine/acetic acid/water 1,250:36:86:56:558 (thin layer chromatography buffer) overnight. After drying, plates were scanned in an Ambis radioscaner system for quantitative analysis.

**Phosphoamino Acid Analysis**—Peptides—Spots of interest were scraped from the plates, extracted twice with 200 μl of peptide electrophoresis buffer, and concentrated to dryness. Residues were treated with 6 M HCl for 90 min at 110 °C. After repeated lyophilization with water and with 50 μl of PAA buffer (acetic acid/pyridine/water 50:5:945 (17)) samples taken up in PAA buffer and aliquots containing comparable amounts of radioactivity (cpm) were loaded on a 20 × 20-cm cellulose plate together with a mix of the three unlabeled phosphoamino acids. Electrophoresis in PAA buffer was at 1,000 V for 50 min. Plates were scanned, and in some cases quantified, in a PhosphorImager (Fuji, BAS 1000).

**Peptide Sequencing**—After recovery from the two-dimensional plates, equivalent peptides (as shown in Fig. 2) were pooled for analysis. In duplicate experiments, peptides were recovered from a preparative two-dimensional map (15 μg of tau). Each peptide was redissolved in 50 μl of 10% acetic acid, loaded onto a Hewlett-Packard miniature biphasic column, and washed with 2% trifluoroacetic acid. Sequencing was performed on a Hewlett-Packard G1005A protein sequencer using methods (version 3.0) and reagents of the manufacturer.

**Radiosequencing of 32P-labeled Peptides**—32P-labeled peptides were covalently coupled to a polyvinylidene difluoride membrane that had been derivatized with alyamine groups (Sequelon-AA; Millipore) in the presence of carbodiimide according to the manufacturer's instructions.
Relationship between Tau Phosphorylation and Phosphoepitopes

Assignment of identities and phosphorylation sites for $^{32}$P-labeled peptides separated by two-dimensional peptide mapping of htau40 protein phosphorylated by PK40

| Peptide no. | NH$_2$-terminal peptide sequence$^a$ | $^{32}$P pmol | Phosphoamino acids | Phosphorylation sites$^f$ |
|-------------|----------------------------------|--------------|--------------------|--------------------------|
| 1           | $^{231}$TPPK—P                    | 2.5          | Ser                | Ser$^{235}$               |
| 2           | $^{199}$S—Y—SP—P                  | <1           | Ser, Thr           | Ser$^{199}$ or Ser$^{202}$, Thr$^{235}$ |
| 3a$^b$      | $^{210}$SR—P$^{2}$LPTTPPTR        | 8            | Ser, Thr           | Thr$^{235}$, Thr$^{237}$ |
| 3b$^b$      | $^{396}$SPVSGDT—PR                | 3            | Ser, Thr           | Ser$^{404}$               |
| 4           | $^{177}$TP—APK—PP                 | 1            | Thr                | Thr$^{151}$               |
| 5           | $^{45}$ESPLQ                      | 10           | Thr, (Ser)$^c$     | Thr$^{27}$                |
| 6           | $^{210}$SR—TP$^{2}$LPTTPPTR      | 10           | Thr                | Thr$^{232}$               |
| 7           | $^{151}$IAT/PR                    | 11           | Thr                | Thr$^{153}$               |
| 8           | $^{199}$SGYS/SPG—PG—PGSR         | 4            | Ser, Thr           | Ser$^{199}$, Ser$^{202}$, Thr$^{205}$ |
| 9           | $^{171}$IPAK—PPA—K                | 1.5          | Ser, Thr           | Thr$^{175}$               |
| 10a$^d$     | $^{171}$IPAK—PPAPK—PPSSE         | 4            | Thr                | Thr$^{173}$, Thr$^{191}$  |
| 10b$^d$     | $^{386}$TDHGAIVYK—PVVSGDT—PR     | 8.5          | Ser                | Ser$^{396}$, Ser$^{404}$ |
| 11          | $^{68}$—PTAEDV                    | 22           | Thr                | Thr$^{69}$                |

$^a$ Assignment of underlined residues is tentative; amino acids in parentheses indicate unusually low recovery, presumably due to contaminating sequences.

$^b$ Initial coupling yield extrapolated back to cycle 0.

$^c$ Underlined residues indicate that assignment is based on inference from a combination of the following criteria: relative migration properties, phosphoamino acid analysis, ERK2 consensus sites, abnormal tryptic cleavage.

$^d$ Peptides tend to colocalize on two-dimensional peptide maps.

$^e$ Phosphoester signals are from unidentified contaminant peptides (not reproducibly observed with peptide 5).

$^f$ Assignment of phosphoamino acid(s) at the site(s) indicated.

Membranes were then washed sequentially with 400 µl of water, 1:1 (v/v) methanol/water, methanol, and water again. Sequencing was performed by automated Edman degradation on an Applied Biosystems model 477A protein sequencer with modified cycles to extract the unform by automated Edman degradation on an Applied Biosystems model 477A protein sequencer with modified cycles to extract the unconverted anilinoheterosilazolinone amino acid derivatives with neat trifluoroacetic acid followed by 1:1 (v/v) ethyl acetate-n-butylchloride containing 0.01% phosphoric acid. Fractions (1 ml) were collected, and radioactivity was determined in a scintillation counter.

RESULTS

Correlations of Phosphorylation Stoichiometry, Gel Mobility, and Immunoreactivity—After incubation with increasing amounts of the ERK2 form PK40 (13, 15) recombinant tau protein showed a spectrum of changes of conformation (as indicated by gel mobility shifts) and of immunological properties. Species with reduced gel mobility became apparent at an average stoichiometry of about 2 mol of PO$_4$/mol of tau, whereas species with maximal mobility shift appeared only at a phosphorylation stoichiometry of ± 7 (Fig. 1). All tau species exhibited maximal gel mobility shift at a stoichiometry of 11.

Tau was probed with mAbs for epitopes that are eliminated by phosphorylation (Tau-1, SMI33) or induced by phosphorylation (PHF-1, SMI31, SMI34). Phosphopeptide and site-directed mutagenesis studies indicated that the reactivity of these mAbs with tau is regulated by one or at most two phosphorylation sites: Ser$^{199}$ and Ser$^{202}$ for Tau-1 (8), Ser$^{396}$ and Ser$^{404}$ for PHF-1 (9), Ser$^{205}$ for SMI33, Ser$^{396}$ for SMI31 (in some instances with a contribution by Ser$^{404}$) and SMI34 (in conjunction with unidentified phosphorylation sites (11, 12)). The SMI mAbs were raised against neurofilaments but cross-react with tau because of the shared sequence motif Lys-Ser-

Assuming that the above epitope assignments were correct, the correlation of the gel mobility state of tau with its immunological reactivity in Fig. 1 would suggest the following.

- Tau species with intermediate gel mobility are already fully phosphorylated at Ser$^{199}$/Ser$^{202}$ and Ser$^{235}$ but not at Ser$^{396}$ or Ser$^{404}$ since they barely react with tau because of the shared sequence motif Lys-Ser-

In some cases criterion 1 did not suggest phosphorylation clearly, possibly due to contamination; however, phosphorylation at the site in question was accepted when all other criteria combined strongly suggested phosphorylation.

Comparative Analysis of Immunological Properties and Phosphopeptides of Tau at Various Stages of Phosphorylation—Superficial examination of the two-dimensional map of high mobility tau species (Fig. 2, panel 2a) showed that most phosphopeptides were already present at some level. Only peptide 10b (Ser$^{396}$/Ser$^{404}$ phosphorylation, Table I) was restricted to

To test the validity of these conclusions derived from immunological analysis, $^{32}$P-labeled mobility isoforms of tau were separately subjected to tryptic phosphopeptide mapping. The major peptides and their phosphorylation sites were identified. As indicated in Fig. 1, tau species of lowest, intermediate, and highest gel mobility were analyzed. Of particular interest were phosphorylation states where two mobility isoforms coexisted (Fig. 1, 2a/2b, 5a/5b).

Identification of $^{32}$P-labeled Peptides and Assignment of Phosphorylation Sites—$^{32}$P-Labeled peptides isolated from two-dimensional peptide maps were identified by Edman sequencing. Assignment of phosphorylation sites (Table I) was by a combination of the following criteria.

1. The absence or very low level of a signal in a sequencing cycle where Ser or Thr would be predicted by the htau40 sequence (19).
2. Phosphoamino acid analysis.
3. Consensus sites of ERK2 (X-Ser/Thr-Pro, X = neutral or basic amino acid (20).
4. Relativemigration patterns of related peptides (diagonal migration shift of multiply phosphorylated peptides on two-dimensional maps (17).
5. Product-precursor relationships between related peptides.
6. Radiosequencing in some cases.
7. Trypsin resistance due to phosphorylation and consequent deviations from the expected electrophoretic mobility due to charges resulting from additional Lys or Arg residues.

In some cases criterion 1 did not suggest phosphorylation clearly, possibly due to contamination; however, phosphorylation at the site in question was accepted when all other criteria combined strongly suggested phosphorylation.
tau species with at least a partial gel mobility shift (Fig. 2, panel 5a). However, no single phosphorylation site fulfilled all
criteria to be solely responsible for any level of gel mobility
alteration: (i) exclusive presence in a lower mobility isoform,
and (ii) stoichiometric presence in reference to the most prom-
inent peptide 1 (Ser235 phosphorylation).

Peptide 1 clearly contained the most preferred site of tau
phosphorylation by PK40 and erk2. According to criteria 1–3, 6,
and 7 (Figs. 2, 3, 4, and 5), Ser235 was assigned as the phosphoryl-
ation site. At an average stoichiometry of 2 PO4/mol of tau,
Ser235 (Fig. 1) was already phosphorylated maximally (Fig. 6),
and it remained the most highly phosphorylated site of tau
up to a stoichiometry of 12. Because of this fortuitous property,
peptide 1 served as an internal reference to assess accurately
the level of phosphorylation of all other sites. Comparison of
immunochemical properties of tau species with their phos-
phoprotein maps showed that Ser235 phosphorylation (see Fig.
6) and elimination of SMI33 immunoreactivity (Fig. 1) were not
correlated. SMI33 reactivity was still strong when Ser235 was
already phosphorylated maximally. Elimination of this epitope
clearly required phosphorylation at other sites.

Peptide 2 was the second most prominent peptide at low
phosphorylation stoichiometries. Because of low recovery, se-
quence information was sparse, but criteria 1–3 suggested
phosphorylation at Ser199 or Ser202, and Thr205, the sites rele-
vant for mAb Tau-1. This assignment was supported further by
relationships with triphosphorylated peptide 8, which con-
tained the same phosphorylation sites: a product-precursor
relationship evident in some experiments, and the diagonal
migration shift (17) relative to the peptide 8 (criteria 4 and 5).
Densitometric analysis of peptides 2 and 8 in Fig. 6 showed
that phosphorylation at Ser199 and Ser202 did not reach the
theoretical level in reference to Ser235 phosphorylation; maxi-
mally only about 40% of the tau species became phosphorylated
on both Ser199 and Ser202 and another 25% at either Ser199 or
Ser202. Nevertheless, the Tau-1 epitope was abolished com-
pletely (Fig. 1), suggesting the involvement of other phos-
phorylation sites.

Peptides 3b and 10b were relevant for the epitopes of PHF-1,
SMI 31, and SMI 34. Although 3b colocalized with unrelated
peptide 3a (see below), criteria 1–3, 6, and 7 identified it clearly
as the Ser404 monophosphorylated peptide cleaved between
Lys395 and unphosphorylated Ser396. It is prominent already at
low stoichiometry of phosphorylation, in contrast to the exclu-
sively Thr-phosphorylated peptide 3a (Fig. 3). Peptide 10b is
the Ser396/Ser404-diphosphorylated homolog of peptide 3b ac-
According to criteria 1–3 and 7 (Figs. 4 and 5). Ser⁴⁰⁴ phosphorylation is apparently required as a prerequisite for Ser³⁹⁶ phosphorylation (criterion 5), since Ser³⁹⁶ monophosphorylated peptides were not recovered. Criterion 4 is not applicable, since the migration relationship between peptides 3b and 10b is atypical due to trypsin resistance induced by Ser³⁹⁶ phosphorylation. Ser³⁹⁶/Ser⁴⁰⁴ diphosphorylation occurred only at relatively high tau phosphorylation stoichiometries associated with partial gel mobility shift (Fig. 2). However, it was not coincident with induction of PHF-1, SMI 31, or SMI 34 immunoreactivity which required full gel mobility retardation (Fig. 1). The SMI mAbs required even higher phosphorylation levels of low mobility tau than PHF-1.

**Other ERK2 Phosphorylation Sites of Tau—Phosphorylation of Thr⁵⁰ (peptide 5), Thr⁶⁹ (peptide 11), and Thr¹⁵³ (peptide 7) was deduced from criteria 1–3, except for peptide 5, where sequencing through the inferred site Thr⁵⁰ was not achieved. Lack of phosphorylation of the ESP motif in peptide 5 is consistent with previous data on ERK consensus sites (20).**

Thr²¹² phosphorylation in peptide 3a was identified according to criteria 1–3, 6, and 7. However, assignment of Thr²¹⁷ phosphorylation in peptide 3a rests on criterion 4 (migration relationship to the homologous peptide 6), since contamination of the sequence data by the more abundant peptide 3b and a trypsin-derived peptide interfered with the interpretation of Edman cycle 8 (Figs. 4 and 5). In the homologous peptide 6 Thr²¹² phosphorylation was suggested by criteria 1–5 and 7 in spite of a weak Thr signal in the relevant Edman cycle (Fig. 4). Thr²¹⁷ was clearly not phosphorylated since it was recovered in the Edman sequence.

Finally, permutations of phosphorylation patterns involving the motif KT¹⁷⁵P/PAPKT¹⁸¹P complicated the assignment of peptides 4, 9, and 10a. Criterion 7 but not criterion 4 was important for the analysis of the phosphorylation state of this motif, since tryptic cleavage was altered by phosphorylation on each site. Peptide 4, tentatively identified by criteria 1–3 and 7 (Table I), occurred only at low levels of tau phosphorylation (≤3 mol of PO₄/mol of tau) and is derived from tau species phosphorylated only at KT¹⁸¹P. At higher levels of tau phosphorylation this peptide was eliminated (Fig. 2; criterion 5) in favor of the Thr¹⁷⁵P/Thr¹⁸¹P-diphosphorylated peptide 10a (criteria 1–3 and 7). Peptide 9 persisted up to the highest levels of phosphorylation (Fig. 2) and represented tau phosphorylation only at KT¹⁷⁵P, but not KT¹⁸¹P, by criteria 1–3 and 7.

**DISCUSSION**

Phosphorylation-dependent monoclonal antibodies are frequently used to assess the phosphorylation state of specific sites of tau from cell cultures or brain. We tested whether immunochemical analysis of tau with several established Ser/Thr-Pro phosphorylation-dependent mAbs reliably reflects the phosphorylation state of specific sites in a background of multiple phosphorylations. For the first time a correlational analysis was used taking advantage of the ability of ERK2 to

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**FIG. 3.** Phosphoamino acid analysis of peptides isolated from two-dimensional phosphopeptide maps. ³²P-Labeled peptides identified on the two-dimensional maps of Fig. 2 were recovered and subjected to PAA (17). Upper panel, PAA of peptides obtained from highly phosphorylated tau (12 mol of PO₄/mol). Quantitative densitometric analysis yielded a higher Ser/Thr ratio for peptide 8 over peptide 2, which assisted in identification as tri- and diphosphorylated forms of the same peptide. Lower panel, PAA of spots 3 and 5, obtained from tau phosphorylated to a lower stoichiometry (4–5 mol of PO₄/mol).

**FIG. 4.** Amino acid yields from Edman sequencing of phosphorylated tau peptides. ³²P-Labeled tryptic peptides 1–11 eluted from two-dimensional maps were directly subjected to NH₂-terminal sequencing. Data of peptides with relatively low recovery (peptides 2, 4, and 5) and many tentative assignments are not shown (see Table I). Ser or Thr residues in parentheses indicate that these amino acids are probably either not or only partially derived from the sequence presented, e.g. in peptide 8 the Ser signal in cycle 5 is believed to be derived from a contamination, as Ser was also elevated in cycles 8, 12, and 17, and other evidence supports phosphorylation of Ser¹⁹⁹. Rarely, trypsin fragments were a source of contamination, e.g. in peptide 3 a Thr signal in critical cycle 8 was contributed by a trypsin fragment as well as by peptide 3b.
Phosphorylation of recombinant tau by the ERK2 form PK40 was not a sequential process, although some sites were preferred. Rather, many sites were phosphorylated concomitantly. Although this does not necessarily predict a similar lack of specificity in biological systems, it fits well into the notion of enormous heterogeneity of tau phosphoisoforms (21) and partial phosphorylation of virtually all Ser/Thr-Pro sites in rat fetal tau to varying degrees.2 In spite of this general trend, the motif KS235P was clearly preferred. This site is believed to be recognized in its unphosphorylated state by the neurofilament mAb SMI33 (11, 12). In our correlational study, however, complete phosphorylation of Ser235 did not suffice to eliminate the SMI33 epitope. Thus, an unphosphorylated KS235P motif is not necessary for mAb binding, and its phosphorylation may be necessary but not sufficient for epitope elimination. Ser199 and Ser202 in conjunction with Thr205, a site previously implied in Tau-1 recognition (10, 22), was the second preferred phosphorylation motif of PK40erk2. Nevertheless, both Ser199 and Ser202 remained partially unphosphorylated in some otherwise highly phosphorylated tau species, although the Tau-1 epitope was abolished. Thus, phosphorylation at Ser199/Ser202 is not strictly required for epitope elimination, in contrast to earlier suggestions based on site-directed mutagenesis studies (8). The complex relationship between the Tau-1 epitope and phosphorylation has also been indicated by a study with multiple tau phosphopeptides (23).

The two examples of mAbs with epitopes eliminated by phosphorylation (Tau-1, SMI33) suggest that interpretations of immunochromen data with this kind of antibody are particularly problematic. Conclusions about the phosphorylation state of specific sites could even be qualitatively incorrect. The failure of SMI33 to report reliably the phosphorylation state of the KS235P motif is particularly compelling; this motif is likely to be directly involved in the binding of mAb SMI33 since it is the only shared sequence motif between tau and neurofilaments, the original antigen of the SMI series of mAbs.

Monoclonal antibodies SMI31/34 and PHF-1 represent cases where the epitope is induced by phosphorylation. Previous work based on site-directed mutagenesis and phosphopeptide studies had defined one or two specific phosphorylation sites as sole determinants of the epitopes (9, 11, 12). However, in this study phosphorylation at those specific sites could be identified in tau species not recognized by these mAbs. The discrepancy was more pronounced with the SMI mAbs than with PHF-1. Hence, phosphorylation at these specific sites may at best be necessary but is not sufficient for epitope formation. However, immunostaining by this type of mAb (epitope induced by phosphorylation) may yield at least qualitatively correct information, since epitope formation has only been observed in conjunction with phosphorylation at the determinant sites. On the other hand, the absence of immunostaining is uninterpretable. Furthermore, a linear relationship between epitope formation and phosphorylation of specific sites should not be assumed.

A new phosphorylation-dependent mAb, AP422, has recently been described to report the phosphorylation state of the DS422P motif in tau (24). Interestingly, only mitogen-activated protein kinase, but not cdk5 or GSK-3, could induce recombinant tau immunoreactivity with this mAb in vitro. However, Ser422 is preceded by an acidic residue and is therefore not necessarily a favored phosphorylation site for ERK2. In our study neither ES46P nor DS422P phosphorylated peptides were recovered, although PK40 was able to induce the AP422 epitope strongly. Thus Ser422 is either only a minor site for

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ERK2, or phosphorylation at this site occurs only at stoichiometric ratios beyond 12.

The precise nature of the relationship between the actual phosphorylation state of tau and the phosphorylation-dependent presentation of epitopes is of importance for the analysis of normal and pathological tau phosphorylation states ex vivo. Immunochemical profiles are often used to evaluate potential biological model systems for PHF-tau-like phosphorylation (e.g., the similar immunochemical properties of tau proteins from fetal brains and AD brains has led to the suggestion that similar neuronal phosphorylation environments exist in these two situations). More direct analyses of tau phosphorylation states are often cumbersome and fraught with technical difficulties, depending on the source of the protein. The present study suggests that the relationship between tau phosphorylation and immunochemical properties is sufficiently complex to place some limits on the use of tau as a reporter protein for physiological phosphorylation environments. An additional restriction arises from evidence that phosphorylation at sites other than Ser/Thr-Pro either modulates proline-directed phosphorylation or alters the phosphorylation/epitope relationship (25-27). PHF-like electrophoretic mobility alterations are also not completely unambiguous; tau species with most Ser/Thr-Pro sites only partially phosphorylated can display full retardation in electrophoretic mobility. Consequently, many alternative phosphorylation patterns must exist which translate into maximally reduced electrophoretic mobility.

In summary, the analysis of tau as a reporter protein for AD-like phosphorylation events is valuable in providing exclusion criteria for model systems, if carried out carefully. A more detailed understanding of normal and pathological neuronal phosphorylation homeostasis will require a comparative analysis of various cell biological circumstances that fulfill the PHF-tau criteria but also match other criteria, such as biological context or regulatory features of kinases/phosphatases in adult central nervous system neurons.

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Table II
Summary of relationships between mAb phosphoepitopes and specific phosphorylation sites

| Phosphorylation site(s) | Stoichiometric phosphorylation | Preference | Relevance for change of mAb reaction | mAb
|------------------------|-------------------------------|------------|-------------------------------------|------|
| Ser396                 | Yes                           | + + +      | +                                  | SMI33|
| Ser199,202,204/Thre205 | No                             | + +        | –                                  | Tau-1|
| Ser396                 | No                             | Least      | +                                  | PHF-1|
| Ser404                 | No                             | +          | (;++?)                             | SMI31a|

a When PK40 is used as the tau kinase, mutation of Ser396 to Ala alone is sufficient to eliminate SMI31 reactivity (12). With crude brain extract, Ser404 has to be mutated also (11).

b A second determinant distant to Ser396 (and Ser404) had already been implicated in tau mutagenesis studies (11, 12).