Protein Sequence and Mass Spectrometric Analyses of Tau in the Alzheimer's Disease Brain*

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Masato Hasegawa‡, Maho Morishima-Kawashima‡, Koji Takio§, Masami Suzuki§, Koiti Tanii§, and Yasuo Ihara‡†

From the ‡Department of Neuropathology, Institute of Brain Research, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan, the §Biomedical Characterization Center, RIKEN, Wako, Saitama 351-01, Japan, and the ¶Division of Biomedical Polymer Science, Institute of Comprehensive Medical Science, School of Medicine, Fujita Health University, Toyoake, Aichi 470-11, Japan

Tau with unusually slow mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis was purified from the Sarkosyl-insoluble pellet of Alzheimer's disease brain homogenates. Such species of tau (PHF-tau) are considered to construct the framework of the sodium dodecyl sulfate-soluble form of paired helical filaments (PHF). Detailed comparison of peptide maps of PHF-tau and normal tau before and after dephosphorylation pointed to three anomalously eluted peaks which contained abnormally phosphorylated peptides, residues 191-225, 226-240, 260-287, and 386-438, according to the numbering of the longest tau isoform (Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D., and Crowther, R. A. (1989) Neuron 3, 519-526). Protein sequence and mass spectrometric analyses localized Thr-231 and Ser-235 as the abnormal phosphorylation sites and further indicated that each tau 1 site (residues 191-225) and the most carboxy-terminal portion of the protein (residues 386-438) carries more than two abnormal phosphates. Ser-262 was also phosphorylated in a fraction of PHF-tau. Modifications other than phosphorylation, removal of the initiator methionine, and N-acetylation at the amino terminus and deamidation at 2 asparaginyl residues were found in PHF-tau, but these modifications were also present in normal tau.

Paired helical filaments (PHF) are unit fibril of neurofibrillary tangles which are formed in rather selected neuronal populations including pyramidal cells in the hippocampus, layers 2 and 4 of the entorhinal cortex, layers 3 and 5 of the association cortex, and relatively large neurons in the nucleus basalis of Meynert, the septal nucleus, the nucleus raphe dorsalis, and the locus ceruleus. These are the subsets of neurons lost during the progression of Alzheimer's disease (AD). Thus, the tangle formation appears to be the final common pathway to neuronal death in these neurons, which in turn is closely related to dementia, the central symptom of AD (1, 2).

Unusual solubility characteristics, in particular, insolubility in sodium dodecyl sulfate (SDS) (3), have prevented rapid progress in the elucidation of the nature of PHF. Using an immunochemical approach with PHF-specific antibodies showed that tau is a major component of PHF (5, 6). Tau is known to be a heat-stable microtubule-associated protein which promotes microtubule assembly and stabilization (7, 8). Human tau cDNA cloning showed the presence of 6 tau isoforms that are alternatively spliced gene products from a single gene (9, 10). There are 29- or 58-amino acid inserts in the amino-terminal portion and a 31-amino acid repeat insert in the microtubule-binding region. Although isoforms with these inserts are expressed only in the adult brain, tau without any inserts, which is the only species in the fetal stage, continues to be expressed throughout life (9, 10).

The carboxy terminal of tau (11, 12) and ubiquitin (13) were sequenced from the SDS-PHF digest (11) or the Pronase-treated PHF fraction (12). The fragmentation of tau was interpreted as follows: the amino-terminal half of the tau in PHF is gradually cleaved off in vitro and the carboxyl third is left behind in SDS-PHF (11). In other words, the carboxyl third of tau is likely to compose the core portion of PHF. The aggregation of tau into PHF appears to precede ubiquitination (14, 15). Thus, it was believed that full-length tau alone could construct the framework of PHF. In fact, Greenberg and Davies (16) and Lee et al. (17) showed that the tau with unusually slow mobilities, called A68 (17, 18) or PHF-tau (19), is the only constituent of SDS-soluble PHF presumably representing an early stage of PHF.

Although there was significant confusion about the nature of PHF-tau, it has now been shown that PHF-tau consists of a hyperphosphorylated species of tau: (i) alkaline phosphatase treatment brought about normal mobilities in SDS-PAGE (17, 20); (ii) its cyanogen bromide fragments gave the amino acid sequences completely homologous to tau (17). Major phosphorylation sites of PHF-tau were claimed to be localized in the middle portion to which a monoclonal antibody, tau 1, specifically binds (tau 1 site) (6, 21), and at the carboxy-terminal portion which polyclonal PHF antibodies recognize (11, 22), and in particular, in the segment KSPV (residues 395-398) which is known to be repeated many times in neurofilament subunits, NF-H and NF-M (17, 23). It is possible that the phosphorylation of tau induces a profound
conformation change by which the tau can self-assemble into PHF. However, it is not clear yet where the abnormal phosphorylation sites in PHF-tau are as compared to normal tau, and whether PHF-tau has any other modification.

In this work we have attempted to determine the sites of abnormal phosphorylation and other modifications, if any, in PHF-tau by protein sequence and mass spectrometric analyses rather than by immunochemical procedures which often bring about significant ambiguities.

EXPERIMENTAL PROCEDURES

Purification of PHF-Tau (A68) from AD Brain—Sarkosyl-insoluble fractions from AD and normal brains were prepared as described previously (5). The Sarkosyl pellets were suspended in a small volume of 50 mM Tris-HCl (pH 7.6), and dissolved with 6 M guanidine HCl for further purification. The guanidine HCl suspension was centrifuged at 500,000 × g for 30 min on a TL-100.3 microcentrifuge (Beckman). The supernatants were treated with iodoacetate after reduction and fractionated on a TSK gel G-3000 SW column (7.8 × 600 mm, Tosoh) equilibrated with 6 M guanidine HCl in 10 mM phosphate buffer (pH 6.0), at a flow rate of 1.0 ml/min. The TSK fractions containing full-length tau with unusually slow mobilities in SDS-PAGE were further purified on an Aquapore RP300 column (2.1 × 30 mm, Applied Biosystems) by HPLC (Hewlett-Packard, Model 1090 M), which was developed with a linear gradient of 20–40% acetonitrile in 0.1% trifluoroacetic acid in 10 min at a flow rate of 0.2 ml/min.

Purification of Normal Tau from Control Brain and Soluble Tau from AD Brain (AD-soluble Tau)—Normal tau from control brain and AD-soluble tau from AD brain were prepared from the supernatants of brain homogenates after centrifugation at 500,000 × g for 30 min, as described previously (11). Crude tau eluted from a phosphocellulose (P11, Whatman) column, after being concentrated with ammonium sulfate, was suspended in 6 M guanidine HCl, and the suspension was fractionated as described above.

Achromobacter lyticus Protease I (API) Peptide Mapping—Purified PHF-tau, AD-soluble tau, or normal tau was divided into halves: one-half was treated with 10 units/ml Escherichia coli alkaline phosphatase (Sigma, type III) for 2 h at 37 °C, as described previously (24), and fractionated on a RP300 column to remove alkaline phosphatase. Equal amounts of untreated and treated tau were digested in 50 mM Tris-HCl (pH 9.0), 2 M urea with API (25) at an enzyme to substrate ratio of 1:100 (w/w) for 15 h at 37 °C. The digests were applied to a Superspher Select B column (2.1 × 125 mm, Merck) and eluted with a linear gradient of 4–48% acetonitrile in 0.1% trifluoroacetic acid in 20 min at a flow rate of 0.2 ml/min.

Amino Acid Sequence and Mass Spectrometric Analyses of the API Peptides—Fractionated peptides were sequenced on an Applied Biosystems 477A Protein Sequencer equipped with an on-line 120A PTH Analyzer or on an Applied Biosystems 473A Protein Sequencer.

Mass spectral analysis was performed on a PE-SCIEX API III Biomolecular Mass Analyzer (triple-stage quadrupole mass spectrometer) equipped with a standard atmospheric pressure ion source (26). Peptides were dissolved in an appropriate volume of 33% isopropanol containing 0.1% trifluoroacetic acid or 0.1% ammonium hydroxide for the positive- or negative-mode analysis, respectively.

Immunological Methods and Antibodies—Electrophoresis, immunoblotting, and enzyme-linked immunosorbent assay were performed as described previously (24).

Antibodies used were Alz 50 (18, 27), anti-human tau (28, 29), antipeptide antibodies to tau C4, C5, C6 (11), BR134 (10), anti-PHF (4, 11, 22, 30), and DF2 (13).

RESULTS

Purification of PHF-Tau (A68), AD-soluble Tau from AD Brain, and Normal Tau from Normal Brain—Large amounts of both anti-PHF immunoreactivities and PHF themselves were recovered into the Sarkosyl-insoluble pellet from AD brain (5). Immunoblot analysis with anti-human tau revealed the presence of abnormal tau with unusually slow mobilities only in the AD Sarkosyl-insoluble fraction (Fig. 1). We could not detect any tau immunoreactivities from an identically prepared fraction from control aged brain. On the other hand, tau in the Tris saline-soluble fraction from AD brain showed the same mobilities and immunoreactivities as that from normal control brain (Fig. 1).

Thus, we purified the normal tau with unusually slow mobilities from the Sarkosyl-insoluble pellet of AD brain. This species of tau was found to be solubilized with 8 M urea or 6 M guanidine HCl. The guanidine HCl extract was fractionated by size on a TSK gel G-3000 SW column (data not shown). The fractions containing the abnormal tau as judged by SDS-PAGE and immunoblotting were further purified by reversed-phase HPLC (RP-HPLC), which gave one major and a few minor peaks (Fig. 2A). Identically prepared fractions from control aged brain contained none or only a trace amount of the corresponding peaks (Fig. 2B). The major peak derived from AD Sarkosyl-insoluble pellet contained the tau which showed (i) unusually slow mobilities in SDS-PAGE and their shift to normal mobilities by dephosphorylation (Fig. 3, A and B) and (ii) the absence of tau 1 immunoreactivities and their marked enhancement after alkaline phosphatase treatment (Fig. 3C). In our hands, thus purified tau from the AD Sarkosyl-insoluble pellet did not always consist of three major polypeptides as described by other investigators (17, 19, 31). Perhaps this may be attributable to a further RP-HPLC purification step employed here.

The early eluting (4.5–6.0 min) peaks in the PHF-tau profile (Fig. 2A) appeared to represent carboxyl terminus-deleted tau, because carboxyl terminus-specific tau antibodies, BR134 and anti-tau C6, barely detected these tau species, and API peptide mapping of these species did not provide the most retarded peak representing a relatively large API peptide, residues 396-438, according to the numbering of the longest human tau isoform (10) (data not shown). The content of these tau species differed from case to case, suggesting that these species are produced during the postmortem period. Thus, the late eluting (6.0–7.5 min) major peak containing the full-length tau was subjected to further analysis. This is defined as PHF-tau here (19). Ubiquitin was contained in the broad peak following the major peak of the Sarkosyl-insoluble tau (see Fig. 2A), as judged by immunoblotting. This broad peak appeared in SDS-PAGE smears in the mid-molecular weight regions, but not in distinct bands (data not shown). The above indicates that the abnormally phosphorylated full-length tau, PHF-tau, is not ubiquitinated.

Normal and AD-soluble tau were prepared from normal
and AD brain, respectively. Final purification was achieved by RPHPLC, which gave almost identical elution profiles for both normal and AD-soluble tau: one major peak that was subjected to further analysis and two minor peaks eluted earlier that also contained truncated forms of tau (Fig. 2, C and D; see above). Thus purified tau from normal and AD brain showed bands with the same mobilities in SDS-PAGE prior to or after dephosphorylation; minimal mobility shifts were observed after dephosphorylation (Fig. 3, A and B). These observations indicate that normal and AD-soluble tau are indistinguishable from each other with respect to elution positions in the RPHPLC profile and mobilities in SDS-PAGE.

Comparison of the Peptide Maps of PHF-Tau, AD-soluble Tau, and Normal Tau—We attempted to analyze PHF-tau, AD-soluble tau, and normal tau systematically to address the issues of (i) whether there is any difference between AD-soluble tau and normal tau, in particular in terms of phosphorylation and other modifications and (ii) where PHF-tau undergoes abnormal phosphorylation as compared with normal or AD-soluble tau; (iii) whether PHF-tau consists of a distinct tau isoform (32, 33); and (iv) whether PHF-tau has unusual post-translational modifications other than phosphorylation. We performed detailed peptide mapping of PHF-tau, AD-soluble tau, and normal tau, and also protein sequence and mass spectrometric analyses of the generated peptides. Because API is highly specific for Lys-X and active even in the presence of denaturant or detergent (14, 25), API digestion should provide a highly reproducible peptide map. If unusual peaks are detected in the HPLC profile, they should represent phosphorylated peptides, modified peptides (API does not cleave Lys-X once the ε-amino group of Lys is modified), or incompletely cleaved peptides, possibly attributable to abnormal phosphorylation and other modifications of adjacent residues (see below). Thus, the search for anomalous peaks in the HPLC profile of PHF-tau or AD-soluble tau as compared with that of normal tau leads to the identification of abnormally phosphorylated or modified peptides. In addition, combined with alkaline phosphatase treatment, this procedure can detect phosphopeptides more easily.

The API digest of PHF-tau, AD-soluble tau, or normal tau with or without prior alkaline phosphatase treatment was fractionated on a Superspher Select B column by RPHPLC (Fig. 4). Approximately 20 peaks were distinguishable and compared between normal and AD-soluble tau or PHF-tau profiles. Normal or AD-soluble tau with or without prior dephosphorylation provided an essentially identical peptide map, strongly suggesting that AD-soluble tau is normal. This assumption was further confirmed by mass analysis of all the peaks in both HPLC profiles (data not shown).

While normal or AD-soluble tau displayed no significant alteration in the peptide map before or after alkaline phosphatase treatment (Fig. 4, C and D), PHF-tau exhibited remarkable changes after its dephosphorylation (Fig. 4, A and B). The two peaks C13 and C20 in the profile of normal or AD-soluble tau were not identified in that of PHF-tau. A very high peak, A5, and an unusually broad peak, A20, were characterized of PHF-tau but never found in normal tau. The alkaline phosphatase treatment of PHF-tau generated two peaks (B13 and B20) which apparently corresponded with C13 and C20 in normal tau, respectively. The observations strongly suggest that these peaks (C13, C20, A5, and A20) contain particular segments which undergo distinct phos-
phosphorylation not found in normal or AD-soluble tau.

These peaks were subjected to amino acid sequence analysis. The peptides in peaks C13 and C20 were determined as SGDR... (191-) and SPVV... (396-), respectively (Fig. 5). A PHF-tau-specific peak A20 gave the major sequence of TDH... (386-) (Fig. 5), indicating that the carboxyl side of Lys-395 remained uncleaved in PHF-tau (Fig. 5). Monitoring by absorbance at 275 nm, we found that an unusually broad peak at 12–13 min contains a tyrosine residue, which is also the case with A20. Rechromatography of the combined fraction, A10-A12, well separated this broad peak (A13; Fig. 3A, inset). Sequence analysis of the broad peak, A13, gave SGD... (191-) as a major signal and TPP... (181-) as a minor signal (Fig. 5).

A very high peak, A6 in the PHF-tau profile, contained three peptides, GQAD... (164-), VAVV... (226-), and IGST... (260-) (Fig. 5). The peptide, VAVV... (226-) was shown, by sequencing, not to terminate at Lys-234, due to failure in cleavage at this lysyl bond. Furthermore, Ser-235 was recovered as a PTH-dehydroalanine derivative in the carboxyl-terminal portion by ISMS. Prior to dephosphorylation, the two PTH-dehydroalanine derivatives in the total absence of PTH-serine, strongly suggesting that Ser-235 was phosphorylated. The minor constituent, IGST... (260-), gave a similar sequencing signal at the 3rd cycle of Edman degradation; Ser-262 was recovered as a PTH-dehydroalanine derivative with little PTH-serine, suggesting that Ser-262 was also phosphorylated.

All of the peaks from PHF-tau were subjected to sequence analysis. The two asparaginyl residues, Asn-157 and -279, were assigned partly as Asp, which was also found in the other two kinds of tau and probably due to partial deamidation (34).

Mass Spectrometric (ISMS) Analysis of API Peptides from PHF-Tau, AD-soluble Tau, and Normal Tau—To further confirm the phosphorylated peptides and also detect other types of modifications, all the peaks from PHF-tau, AD-soluble tau, and normal tau were subjected to ion-spray mass spectrometry (ISMS) on a PE-SCIEX API III Biomolecular Mass Analyzer. Mass analysis of A5 gave signals corresponding to molecular masses of 1683.0 (1523.0 + 80 × 2), 1126.6, and 940.6 (860.6 + 80 × 1), indicating that residues 226–240 (calculated molecular mass = 1523.8) contain two phosphates, and residues 260–267 (calculated molecular mass = 861.0) have one phosphate (Fig. 6, Table I). In contrast, the corresponding peptides from normal tau or AD-soluble tau only gave signals of nonphosphorylated peptides (Fig. 6, Table I).

Analysis of B6 from dephosphorylated PHF-tau gave signals derived from a molecular mass of 1045.6 (965.6 + 80 × 1), indicating that residues 226–234 contain one phosphate, and thus Thr-231 remained phosphorylated even after alkaline phosphatase treatment of whole PHF-tau (Fig. 6, Table I). These observations, together with the amino acid sequence data, showed that Thr-231, Ser-235, and Ser-262 are phosphorylated. The peptides phosphorylated at Thr-231 or Ser-262 are hardly detected in normal tau or AD-soluble tau (Fig. 6, Table I).

Analyses of peaks B8 and B9, which showed a significant increase after alkaline phosphatase treatment, gave signals corresponding to the molecular masses of peptides AKTDHGAIVYK and TDHGAIVYK. They are probably produced by the restored API action at the Lys-Ser bond in TDH... (A20) after alkaline phosphatase treatment.

We also attempted to analyze the tau 1- and carboxy-terminal sites by ISMS. Prior to dephosphorylation, the two relatively large peptides (A13 and A20) provided no significant signals for some unknown reasons. This may be a characteristic of the two peptides, because an electrospray MS (TSQ 700, Finnegan MAT) also gave no significant signals. After dephosphorylation, the tau 1 site (B13) gave signals derived from a molecular mass of 3533.4 (3453.4 + 80 × 1) (Table I). Similar results on the most carboxy-terminal portion were obtained. Two sets of signals, corresponding to molecular masses of 4328.4 and 4408.9 (4328.9 + 80 × 1) were observed by the analysis of B20 (Table I). This indicates that a fraction of residues 396–438 bears one phosphate even after dephosphorylation. Similarly, peak C20 gave two signals corresponding to molecular masses of 4326.7 and 4407.9 (4327.9 + 80 × 1), indicating that a fraction of the peptide is monophosphorylated (Table I). However, this phosphopeptide was easily removed with alkaline phosphatase, as confirmed by ISMS.
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Thus, the location of the remaining phosphate in PHF-tau probably differs from that found in normal tau.

All the remaining fractions were subjected to ISMS which provided signals corresponding to the majority of API peptides spanning full-length tau (Fig. 5, Table I). The analysis was clearly shown in the API digest of PHF-tau: (i) the presence of two amino-terminal peptides with no insertion and a 29-amino acid insert (residues 45–73; 10) and (ii) the presence of both three- and four-repeat-specific peptides (9, 32, 33, 35); and (iii) the absence of post-translational modifications with relatively large molecular mass (for example, glycosylation) other than phosphorylation, removal of the initiator methionine, and N-acetylation of Ala-2 at the amino terminus of tau as a co-translational modification.

**DISCUSSION**

One of the unexpected results obtained in the present work is that normal tau and AD-soluble tau are essentially the same with respect to phosphorylation, other modifications, and presumably isoform abundance (see below). Thus, the abnormal species of hyperphosphorylated tau (PHF-tau) is almost undetectable in the cytosolic compartment of AD brain, although we cannot completely rule out the possibility that a trace amount of such tau is soluble. It remains unknown whether the abnormal species of tau is generated before or after PHF formation. From the above result, it is still possible to postulate that its unusual configuration within PHF allows abnormal phosphorylation. This assumption may be compatible with the observation that the portions surrounding microtubule-binding domains implicated in the PHF formation (19, 33) are highly phosphorylated.

Our sequence and ISMS analyses of API peptides from PHF-tau covered 80% of the full-length tau (Fig. 5). Missing portions are expected to yield rather short, very hydrophilic peptides which might have been lost in the breakthrough peak except for the amino-terminal 58-residue insert (residues 74–102). Identification of two types of the amino-terminal insert-specific peptides (A16, 103–130; A17, 68–73 103–130) and the three- and four-repeat-specific peptides (A14, 299–311; A17, 68–73 103–130) and the three- and four-repeat-specific peptides (A14, 299–311; A17, 299–317; A11, 306–311; A16, 306–317) (Table I) suggests that there may be four major isoforms in PHF-tau, and isoforms having a 58-amino acid insert in the amino terminus (27) may be the least abundant. Similar results were also obtained on normal and AD-soluble tau. In addition, there was no significant difference in the heights of peaks 11 and 14 which contained three- and four-repeat-specific peptides, respectively (Fig. 4). These observations suggest that PHF-tau is not distinct from normal tau with respect to isoform predominance. Our observations are consistent in part with the previously reported immunochemical results (36, 37).

Thus far, several potential phosphorylation sites of PHF-tau were immunochemically determined. Ser-396 (17) and Ser-404 (22) were claimed to be potential phosphorylation sites of PHF-tau. Particular portions, residues 189–207 (tau 1 site) (21), and residues 402–420 (22) were also claimed to be abnormal phosphate acceptors. In this study, we have directly identified four abnormal phosphorylated peptides by a protein chemical approach, residues 191–225 (fragment 1), 226–240 (fragment 2), 260–267 (fragment 3), and 386–438...
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FIG. 6. ISMS of peak 5 (I) and peak 6 (II) from PHF-tau (A and B) and AD-soluble tau (C). I, (M + 2H)+ ion at m/z 842.5 (VAVVRTPPKSPSSAK) containing two phosphates and (M + H)+ ion at m/z 941.5 (IGSTENLK) containing one phosphate were detected in addition to the common (M + 2H)+ ion at m/z 564.3 in peak A5 from PHF-tau. These signals were not detected in peak B5 from dephosphorylated PHF-tau and peak C5 from normal tau. II, (M + H)+ ion at m/z 861.5 (nonphosphorylated peptide IGSTENLK) was also detected in peak A6 from PHF-tau. (M + 2H)+ ion at m/z 523.8 (VAVVRTPPK) containing one phosphate was detected in peak B6 from dephosphorylated PHF-tau, but only trace amounts of the ions were detected in C6 and D6 (not shown) from normal and dephosphorylated normal tau.

(fragment 4). These peptides are characterized by: (i) anomalous elution position in the HPLC profile (fragments 1-4); (ii) unusual resistance to API cleavage at Lys-Ser unless dephosphorylated (fragments 2 and 4); (iii) unusually broad peak on a reversed-phase column (fragments 1 and 4); and (iv) unusual resistance to dephosphorylation with alkaline phosphatase (fragments 1, 2, and 4).

Thr-231 was unambiguously identified as an abnormal phosphorylation site and this site was unusually resistant to dephosphorylation. As for Ser-235, there remains some uncertainty whether the phosphate on residue 235 is present only in PHF-tau, because we failed to recover SPSSAK (235-240), with or without phosphate, from normal or AD-soluble tau digests. However, it is most likely that the phosphorylation on Ser-235 is specific for PHF-tau, because (i) the peptide, residues 226-240 containing two phosphates, is detected only in PHF-tau digest but not in normal or AD-soluble tau digests. However, it is most likely that the phosphorylation on Ser-235 is specific for PHF-tau, because (i) the peptide, residues 226-240 containing two phosphates, is detected only in PHF-tau digest but not in normal or AD-soluble tau digest; and (ii) because the phosphate on Thr-231 does not affect API cleavage (see Fig. 5 and Table I), it is reasonable to speculate that the other phosphate on Ser-235 in residues 226-240 must have prevented effective API action at the carboxyl side of Lys-234. We also found that a fraction of residues 260-267 is phosphorylated in PHF-tau (Table I). The corresponding fragment from normal tau or AD-soluble tau was never phosphorylated. Thus, Ser-262 in the first microtubule-binding domain is also the site for abnormal phosphorylation, which characterizes a fraction of PHF-tau. These phosphorylation sites exactly determined here have not been reported so far in PHF-tau.

Fragment 1 containing the tau 1 site (residues 191-224) and fragment 4 (residues 386-438) from PHF-tau showed characteristic broad peaks in the HPLC profile (Fig. 4). Both relatively large peptides did not provide clear mass signals unless dephosphorylated. Only after dephosphorylation did the above two fragments provide signals corresponding to the monophosphorylated former peptide (fragment 1) and a mixture of monophosphorylated and unphosphorylated latter peptides (fragment 4), respectively. We speculate based on the circumstantial evidence that the undetectability of mass signals corresponding to fragments 1 and 4 prior to alkaline phosphatase treatment is due to their hyperphosphorylation, although we cannot completely eliminate a possibility other than hyperphosphorylation. Thus it is most likely that these fragments carry more than two phosphates.

The abnormal phosphorylation sites determined here are not found in the alternatively spliced inserts but in the common portions shared by all the isoforms. They are localized at both amino- and carboxyl-terminal sides of the micro-
The amino terminus is acetylated (Ac) Ala-2, which is also found in normal or AD-soluble tau.

Four-repeat-specific peptides. Cysteine is carboxymethylated (Cm).

Residues 226-234 hear a phosphate even after dephosphorylation.

A fraction of fragment 4 is monophosphorylated in AD-soluble or normal tau.

Fragments 1-3 are not phosphorylated in AD-soluble or normal tau.

Residues 226-240, fragment 2, are phosphorylated.

A fraction of residues 260-267, fragment 3, is phosphorylated.

Multiply charged signals are calculated to show (M + H)**.

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<td>1122.6</td>
<td>1133.2</td>
<td>TDHGAIYK (356-368)</td>
</tr>
<tr>
<td>B9</td>
<td>2166.5</td>
<td>2167.2</td>
<td>DQGGYTHQEQGDTDAGLK (25-44)</td>
</tr>
<tr>
<td>B12</td>
<td>522.3</td>
<td>522.6</td>
<td>LDYF (344-347)</td>
</tr>
<tr>
<td>B13</td>
<td>522.3</td>
<td>522.6</td>
<td>LDYF (344-347)</td>
</tr>
<tr>
<td>C20</td>
<td>4329.4</td>
<td>4328.8</td>
<td>SPVVSGDTPRHLSNVSSTGSIDMSDPSQLATLAEVSASLAK (396-438)</td>
</tr>
<tr>
<td>C20</td>
<td>4409.9</td>
<td>4408.9</td>
<td>SPVVSGDTPRHLSNVSSTGSIDMSDPSQLATLAEVSASLAK (396-438)</td>
</tr>
</tbody>
</table>

** Multiply charged signals are calculated to show (M + H)**.
° Asn-167 and Asn-279 are partly deamidated, which is also the case in normal or AD-soluble tau (data not shown).
°° Residues 260–267, fragment 2, are phosphorylated.
°°° A fraction of residues 260–267, fragment 3, is phosphorylated.
°°°° Cysteine is carboxymethylated (Cm).
°°°°° Four-repeat-specific peptides.
°°°°°° Three-repeat-specific peptides.
°°°°°°° No significant signals.
°°°°°°°° Amino-terminal-insert-deleted peptide.
°°°°°°°°° Amino-terminal 29-residue insert-specific peptides.
°°°°°°°°°° The amino terminus is acetylated (Ac) Ala-2, which is also found in normal or AD-soluble tau.
°°°°°°°°°° Residues 226-234 bear a phosphate even after dephosphorylation.
°°°°°°°°°°° A fraction of fragment 4 (residues 396-438) also bears one phosphate even after dephosphorylation.
°°°°°°°°°°°° Fragments 1-3 are not phosphorylated in AD-soluble or normal tau.
°°°°°°°°°°°°° A fraction of fragment 4 is monophosphorylated in AD-soluble or normal tau.
°°°°°°°°°°°°°° The phosphate in fragment 4 from AD-soluble or normal tau is easily removed.
Tubule-binding domains. Overall, PHF-tau is abnormally phosphorylated at more than 7 sites in the whole molecule, while normal or AD-soluble tau contains at least one phosphate in the carboxyl-terminal portion (Table I).

The unusual solubility characteristics of PHF suggest the presence of post-translational modifications other than phosphorylation (3). These include N-γ-glutamyl) lysine cross-linkage (3), deamination (38), and N-methylation of the lysine residue (12). In identification of the post-translational modifications including the above candidates, ISMS should be very powerful. In this study, ISMS has shown that normal tau, AD-soluble tau, and PHF-tau begin with acetylated alanine but not with initiator methionine with or without acetylation (see Table I). Except for this modification, ISMS of post-translational modifications other than phosphorylation.

Thr-231
Protein kinase(s) may be involved in the phosphorylation of tau in the interphase microtubular network. It may be that cdc2 kinase, a threonine or serine residue is preceded by a basic residue, is the location of nonphosphoryl modification (39), while normal or AD-soluble tau contains at least one phosphorylation site. It is not possible to specify the kinase(s) involved in the phosphorylation of tau.

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REFERENCES