The amino acid sequences are known for human, mouse, and phosphorylated. These data indicate that the 93-amino acid region encompassed a 93-amino acid region necessarily at the KSPV site.

which recognize PHF-tau and peptides phosphorylated porcine tau even though the KSPV sequence was not was bovine tau proteins (Goedert et al., 1988; Himmler et al., 1989). The serine homologous with S404 of human r441 was phosphorylated on bovine and porcine tau and up to two phosphates were present on a peptide of amino acids 182–240 of bovine tau (193–251 of human r441). The serine within the KSPV motif was not phosphorylated on bovine or porcine tau. PHF-tau fragments, isolated from pronase-treated PHFs encompassed a 93-amino acid region within the microtubule binding domain. Enzymatic digestion and mass spectrometric analysis showed no phosphate was present and a second carboxyl terminus was identified at E380. Antibodies T3P and SMI34, which recognize PHF-tau and peptides phosphorylated at the sequence KSPV, both reacted with bovine and porcine tau even though the KSPV sequence was not phosphorylated. These data indicate that the 93-amino acid sequence of F5.5 tau from PHFs is not phosphorylated at the sequence KSPV, both reacted with bovine and porcine tau. Antibodies T3P and SMI34 react with phosphorylated epitopes that are not unique to PHF-tau and that are not necessarily at the KSPV site.

Tau protein is a phosphorylated neuronal microtubule-associated protein. Tau protein is also present in the major pathological lesions of Alzheimer's disease in an insoluble hyperphosphorylated state as paired helical filaments (PHFs). We have investigated the phosphorylation state of control tau and a fragment of PHF-tau. Tau samples were digested with protease, separated by reversed-phase high-performance liquid chromatography, and analyzed by mass spectrometry and Edman microsequencing. The serine homologous with S404 of human r441 was phosphorylated on bovine and porcine tau and up to two phosphates were present on a peptide of amino acids 182–240 of bovine tau (193–251 of human r441). The serine within the KSPV motif was not phosphorylated on bovine or porcine tau. PHF-tau fragments, isolated from pronase-treated PHFs encompassed a 93-amino acid region within the microtubule binding domain. Enzymatic digestion and mass spectrometric analysis showed no phosphate was present and a second carboxyl terminus was identified at E380. Antibodies T3P and SMI34, which recognize PHF-tau and peptides phosphorylated at the sequence KSPV, both reacted with bovine and porcine tau even though the KSPV sequence was not phosphorylated. These data indicate that the 93-amino acid sequence of F5.5 tau from PHFs is not phosphorylated at the sequence KSPV, both reacted with bovine and porcine tau. Antibodies T3P and SMI34 react with phosphorylated epitopes that are not unique to PHF-tau and that are not necessarily at the KSPV site.

Tau protein is a microtubule-associated protein that induces microtubule assembly and stabilizes microtubules in vitro (Weinberger et al., 1975; Drubin and Kirschner, 1986). The amino acid sequences are known for human, mouse, and bovine tau proteins (Goedert et al., 1988; Lee et al., 1988; Himmler et al., 1989), but not for porcine tau. Six human tau isoforms have been identified, varying in length from 352 to 441 amino acids for the shortest isoform (r352) to the longest isoform (r441) (Goedert et al., 1989). These isoforms differ from each other depending on whether either a 29- or 58-amino acid insert is present after K44 or a 31-amino acid insert is present after K216 of r352, in the tubulin-binding region (Goedert et al., 1989). Three tandem repeat regions are present in the tubulin-binding region of r352, and the presence of the insert in this region results in four tandem repeats, referred to as 3-repeat tau and 4-repeat tau, respectively.

Tau protein is usually phosphorylated, introducing further heterogeneity (Lindwall and Cole, 1984; Butler and Shelanski, 1986). The sites phosphorylated in vitro by calcium-calmodulin-dependent protein kinase (Steiner et al., 1990), tau protein kinase (Ishiguro et al., 1991), protein kinase C (Correas et al., 1992), mitogen-activated protein kinase (Gustke et al., 1992), proline-directed protein kinase (Vulliet et al., 1992), and cAMP-dependent protein kinase (Scott et al., 1993) have been identified. The presence of phosphate groups on tau protein tends to decrease its ability to promote microtubule assembly (Lindwall and Cole, 1984; Hoshi et al., 1987; Yamamoto et al., 1985) and decreases the elasticity of tau (Hagedorn et al., 1989). The locations of endogenously phosphorylated sites have not been precisely defined until recently. Hasegawa et al. (1992) have identified sites in both normal and PHF-tau by direct chemical analysis and have shown that at least three sites are phosphorylated on PHF-tau but not normal tau.

Tau protein is the only protein confirmed to be present in the paired helical filaments (PHFs) of neurofibrillary tangles and neuritic plaques (Wischik et al., 1988; Kondo et al., 1988), important pathological lesions of Alzheimer's disease (Caputo and Salama, 1989; Braak and Braak, 1991). The tau protein that is associated with PHFs appears to be ubiquitinated (Cole and Timiras, 1987; Grundke-Iqbal et al., 1988) and aberrantly phosphorylated, with about four times as much phosphate present compared to control human tau (Ksiezek-Reding et al., 1990). PHF-tau may also be truncated (Kondo et al., 1988; Brion et al., 1991).

Prior to the work of Hasegawa et al. (1992), the locations of phosphorylation sites on either normal tau or PHF-tau had not been precisely determined, although attempts have been made to map these sites using antibodies. For example antibody T3P was raised to a synthetic peptide corresponding to amino acids 389–402 of r441 phosphorylated at S396 (Lee et al., 1991). T3P reacts with PHF-tau but not normal tau, suggesting that S396 is phosphorylated in Alzheimer's disease. This serine occurs in the sequence, KSPV. This KSPV sequence also occurs in neurofilament peptides and antibody SMI34 (also referred to as 07-5) reacts with the phosphorylated version of neurofilaments and reagents with PHFs (Sternberger et al., 1985). Therefore, it too may recognize PHF-tau phosphorylated at S396 within the KSPV motif. This serine is near the tubulin-binding region of tau, and phosphorylation near this region has been suggested to cause a conformational change in this region which is required for SMI34 reactivity (Lichtenberg-Kraag et al., 1992).
Another phosphorylation site in PHFs is postulated to be in or near amino acids 189-207 of r441, the epitope for antibody tau-1 (Kosik et al., 1988). Tau-1 reacts with PHF-tau or tangles only after they are dephosphorylated (Wood et al., 1986; Grundke-Iqbal et al., 1986b; Lee et al., 1991). The phosphate appears to occlude the antibody binding site or possibly induce a conformational change that the antibody does not recognize. Recently antibody AT8 was described, whose reactivity with PHF-tau seems to be localized to the phosphate that interferes with tau-1 binding (Biernat et al., 1992).

The role of tau phosphorylation in the formation of PHFs is unknown. For example, it is not known whether the presence of phosphates at specific positions on tau converts tau into PHFs. This knowledge, however, could be valuable for designing therapeutic interventions for Alzheimer’s disease. One way to approach this question is to determine whether the aberrant phosphorylation sites are unique to PHF-tau or whether they occur on tau molecules such as bovine or porcine tau, which are not known to form PHFs. Another issue that must be resolved in addressing this question is whether the antibodies that have been used to postulate the locations of specific tau phosphorylation sites are specific for phosphate at one particular location on tau or whether they can react with phosphates at several different locations. In the present study we address these issues by using mass spectrometry and Edman microsequencing to identify specific phosphorylation sites on bovine and porcine tau, as well as on a PHF-tau fragment. This fragment, isolated in the F5.5 fraction, binds tightly to PHFs and contains part of the tubulin-binding region of tau (Wischik et al., 1988). Several antibodies that react with PHF-tau in a phosphate-dependent fashion were also evaluated with these control tau protein preparations in order to determine whether they react with the phosphorylation sites that were identified on tau by direct chemical analysis.

**MATERIALS AND METHODS**

**Bovine and Porcine Tau Proteins—**Microtubules were purified from porcine brain and bovine brain using the large-scale method described by Murphy (1982). A phosphate inhibitor mixture consisting of 25 mM 9-glycerophosphate and 0.2 mM sodium orthovanadate was included in all buffer solutions. Tau was extracted from the microtubules using method three of Grundke-Iqbal et al. (1986a) and was further purified using exclusion HPLC on a TSKgel G3000SW column pre-equilibrated in 50 mM Mes, pH 6.25, 100 mM NaCl, 5 mM diithreitol.

**PHF-Tau Protein Fragment (F5.5 Tau)—** Pronase-treated PHF-tau proteins were extracted with formic acid, yielding the F5.5 fraction as described previously (Wischik et al., 1988). This F5.5 (formic acid extract) fraction contains a fragment of tau protein from the tubulin-binding region (referred to as F5.5 tau in this study). Two F5.5 tau samples were prepared from brains of two severe Alzheimer cases, referred to as 76 and 77 and described elsewhere (Harrington et al., 1991; Muttafova-Ladinska et al., 1992). Formic acid was used at 88% to prepare the first sample (case 76) and at 2% for the second sample (case 77). The F5.5 samples were provided by Dr. C. Wischik.

One F5.5 sample (case 76) was further purified by affinity chromatography. A 200-μl aliquot was applied to an affinity column (monoclonal antibody 6.423-linked CNBr-Sepharose), which binds with F5.5 tau (Wischik et al. 1988). The column was washed with 40 mM Tris, pH 8.0, and the sample was eluted from the column with 40 mM acetic acid, pH 3.8, followed by a second elution step employing 40 mM Tris, 0.5 mM sodium chloride, 0.65% Tween. By amino acid analysis the yield of purified protein was 20% of the original sample. No protein was recovered in the second elution step.

**Peptide Mapping of Bovine, Porcine, and Human Tau Proteins—** A 100-μg aliquot of bovine tau, 30 μg of porcine tau, and 30 μg of affinity-purified F5.5-tau were digested with asp-N protease (Boehringer Mannheim, sequencing grade) at an enzyme-to-substrate ratio of 1:100 (w:w), in 100 mM ammonium hydrogen carbonate, pH 7.8, for 16 h at 37 °C. The bovine tau digestion products were separated on a Pharmacia SMART system using a URFC C2/C18 PC 3.3/3 column. Eluent A was 0.1% trifluoroacetic acid (Pierce HPLC/SpectroGrade) in water, and eluent B was 90% acetonitrile (far UV grade) and 0.1% trifluoroacetic acid in water. Peptides were eluted using the following conditions: 3% eluent B, 0-10 min; 3-50% B, 10-60 min; 50-90% B, 60-100 min; 90% B, 100-110 min; 90-3% B, 110-135 min.

Peptide fragments in the porcine tau and F5.5 tau digests were separated using a Waters HPLC system with two 510 pumps, a 490E UV detector and Maxima software, and a Waters DELTA 150 C18 30 A column. The same sequence of eluent samples was run as described above for the SMART system. A 100-μg aliquot of F5.5 tau (case 77) was digested with trypsin (Worthington) at an enzyme-to-substrate ratio of 3:100, in 100 mM ammonium hydrogen carbonate, pH 8.5, for 2 h at 37 °C. The resulting tryptic peptides containing the sites that were identified on tau by direct chemical analysis were submitted for Edman sequencing.

**Antiserum Preparation—** Fast atom bombardment mass spectrometry was performed on a VG ZAB2-SE mass spectrometer, using a thiglycerolglycerol cryo-matrix (1:1, v/v) (Sigma) containing 1% trifluoroacetic acid. Electrospray mass spectrometry was measured using a VG instruments Bio-Q mass spectrometer. Samples were redissolved and introduced into the source in methanol (Analar grade):water (1:1, v/v) containing 1% acetic acid (Analar grade).

**Amino Acid Sequencing and Derivatization of Phosphorylated Peptides with Ethanol—** Amino-terminal sequence analysis was measured on an ABI 475 gas-phase sequencer and the phenylthiodyantoin amino acids were measured with an on-line ABI 120 analyzer. Peptides containing phosphorylated residues were derivatized with ethane thioldiol (Aldrich Chemical Co.) by the method of Meyer et al. (1986). The derivatized amino acid was detected using Edman sequencing.

**Alkaline Phosphatase Treatment—** Tau proteins were treated with alkaline phosphatase where indicated, prior to immunoassay. Ten μl of tau plus 1 unit of Escherichia coli alkaline phosphatase (Sigma) in 45 μl of 0.1 M glycine buffer, pH 10.5, 1 M MgCl₂, 0.1 M phenylmethylsulfonyl fluoride, and 5 mM benzamidine was incubated for 6 h at 37 °C. One unit of phosphatase was then added along with 1 μl of 200 mM phenylmethylsulfonyl fluoride and 5 μl of 1 M benzamidine and incubated for an additional 18 h. 5 μl of bovine tau was combined with 5 units of calf intestine alkaline phosphatase (Boehringer Mannheim, molecular biology grade) in 50 μl of 30 mM ethanolamine buffer, pH 7.6, with 3 M NaCl, 1 mM MgCl₂, and 0.1 mM ZnCl₂, further diluted to 300 μl with 50 mM Tris buffer, pH 8, and incubated overnight at 37 °C.

**Immunoblotting—** Aliquots of the same bovine and porcine tau protein samples that were subjected to mass spectrometry were diluted 1:200 in Tris buffer, pH 7.4 (3 μg of tau-1 or 50 μg sodium dodecyl sulfate), pH 9.6 (bovine tau), and analyzed by ELISA as previously described (Caputo et al., 1992b). Control wells for T3P and tau-1 antibodies were plated with 0.7% bovine serum albumin in phosphate-buffered saline, and control wells for SM34 samples were plated with 0.2% non-fat dry milk in borate-buffered saline. The color reagent used was 2,2'-azino-di-(3-ethylbenzthiazoline-2-sulfonic acid) and color development was allowed to proceed for up to 60 min. Antibody SM34 was purchased from Stemmerber Monoclonals Inc., and antibody tau-1 was generously provided by Dr. Lester I. Binder. Antibodies tau-14, which recognizes amino acids 141-178 of ~441 independent tau sequences, was generously provided by Dr. Virginia Lee. Statistical significance was determined using the Student’s t test.

**RESULTS**

**Proteolysis, rp-HPLC, and Mass Spectrometry of Bovine and Porcine Tau Proteins—** The asp-N digestion products of bovine tau were separated into ten fractions by rp-HPLC (Fig. 1). Each fraction was analyzed by mass spectrometry, and the position for each peptide in the bovine tau sequence...
products were separated by rp-HPLC and analyzed by mass to S393 of bovine tau (S404 of human 7441) based on the peptide containing bovine amino acids 396-406. This result yielded masses together with limited Edman sequencing (Table I). Coverage of the 4-repeat bovine tau sequence from this mapping experiment was approximately 60% (Fig. 2).

Two phosphorylated peptides were identified in this bovine tau digest (Table I). One phosphorylated peptide was present in fraction 2 and corresponded to amino acids 391-406 of 4-repeat bovine tau (402-417 of human r441). The mass spectral data for this peptide indicated that it was present only as the monophosphorylated peptide (Fig. 3A). This peptide contains five amino acids, DTSPR, of the parent peptide. The peptide as described for bovine tau, above. The peptide containing amino acids 394-408 of human r441 which contains the KSPV motif was recovered in only the nonphosphorylated form as was the case with bovine tau. No peptide containing the sequence corresponding to human r441 amino acids 193-251 was assigned by matching calculated and experimentally derived masses together with limited Edman sequencing (Table I). Coverage of the 4-repeat bovine tau sequence from this mapping experiment was approximately 60% (Fig. 2).

Two phosphorylated peptides were identified in this bovine tau digest (Table I). One phosphorylated peptide was present in fraction 2 and corresponded to amino acids 391-406 of 4-repeat bovine tau (402-417 of human r441). The mass spectral data for this peptide indicated that it was present only as the monophosphorylated peptide (Fig. 3A). This peptide contains five potential phosphate acceptor sites (5 serine and 2 threonine residues). To identify the specific residue within this peptide that was phosphorylated, the fraction containing this peptide was subjected to tryptic digestion and reanalyzed by FAB mass spectrometry. A protonated ion of mass 1101.6 Da was observed, corresponding to the nonphosphorylated peptide containing bovine amino acids 396-406. This result indicates that the phosphorylation site was within the first five amino acids, DTSPR, of the parent peptide. The peptide of bovine amino acids 391-406 was dehydrated, derivatized with ethane-thiol to form an ethylcysteine residue, and Edman-microsequenced. The phosphorylation site was assigned to S393 of bovine tau (S404 of human r441) based on the elution of phenylthiohydantoin-ethylcysteine at the corresponding Edman cycle. The identification of nonphosphorylated peptide 396-406 indicates that S405 of bovine tau (S416 of human r441) was not phosphorylated in vivo. This site is known to be phosphorylated in vitro by calcium-calmodulin-dependent protein kinase (Steiner et al., 1992) and cAMP-dependent protein kinase (Scott et al., 1992) resulting in a shift in tau mobility upon electrophoresis.

The second phosphorylated peptide was recovered in fraction 10 (Table I) and corresponds to amino acids 182-240 of bovine tau (193-251 of human r441). Based on mass spectral data, this peptide existed in the nonphosphorylated, monophosphorylated, and diphosphorylated states (Fig. 4). Too little sample remained of this peptide to allow precise assignment of the phosphorylated residues. No other peptide identified in the bovine digest was phosphorylated (Table I), including the peptide containing amino acids 376-390 of bovine tau (387-401 of human r441). This peptide was isolated in fraction 5 and contains the sequence KSPV. The mass spectrum for this peptide is shown in Fig. 3C.

Porcine tau was also digested with asp-N and the digestion products were separated by rp-HPLC and analyzed by mass spectrometry and limited Edman sequencing. The sequence coverage was 50% of the human 4-repeat (r441) tau sequence. One phosphorylation site was identified, which was located on the peptide corresponding to amino acids 402-417 of human r441 (Table II). The monophosphorylated and nonphosphorylated forms of this peptide were detected (Fig. 3B). A serine in porcine tau homologous with S404 of human r441 was determined to be the residue phosphorylated in this peptide based on the analysis of the tryptic digest of this peptide by FAB mass spectrometry, as well as the Edman microsequencing results of the ethane-thiol-derivatized peptide as described for bovine tau, above. The peptide containing amino acids 394-408 of human r441 which contains the KSPV motif was recovered in only the nonphosphorylated form as was the case with bovine tau. No peptide containing the sequence corresponding to human r441 amino acids 193-251

![Graph](image-url)
was detected, probably because electrospray mass spectrometry was not used with any HPLC fraction from the digest of porcine tau.

In addition, a peptide from the digest of porcine tau corresponding to amino acids 358-379 (human 7441) was detected. This peptide ends in arginine and was therefore not expected to be produced by asp-N cleavage. Whether this carboxy-terminally truncated species was generated during isolation or represents a naturally occurring species is not clear. However, it should be noted that the PHF-tau fragments contains a species which is terminated at E380, 1 residue downstream from this terminus (see below). Also Hasegawa et al. (1992) report that their preparation of PHF-tau contains a carboxy-terminally truncated species as determined by a lack of reactivity with carboxyl-terminally specific antibodies BR134 and C6. While it was not possible to quantitate the levels of this species accurately, it represents a minor component (less than 10% of native tau in our sample). There were barely detectable levels of this species in the bovine tau digests as determined by mass spectrometry (data not shown).

Mass Spectrometry of the PHF-Tau Fragment—Affinity-purified F5.5-tau was digested with asp-N protease, and the digestion products were separated by rp-HPLC (Fig. 5). The peptides that were identified in these fractions by mass spectrometry and Edman sequencing covered 95% of a 93-amino acid region from amino acid 299 to 391 of human r441 (Table III). Fractions 1–5 contained peptides with overlapping sequences corresponding to amino acids 50–93 of this sequence. Fraction 1 contained a peptide corresponding to amino acids 50–59. A similar peptide was identified in fraction 2, except that it was one mass unit higher, suggesting deamidation of glutamine. This deamidation may have resulted from extraction of the PHF preparation with 88% formic acid to isolate F5.5 tau.

The 93-amino acid region terminates with the sequence DHGAE. The peptides in fractions 3 and 5 ended at residue 82 with the sequence LTFRE. Both of these termini end with glutamic acid and were not predicted from asp-N cleavage. A peptide was recovered in fraction 6 which contained, based on Edman sequencing, amino acids 1–44 of the 93-amino acid region from 4-repeat tau (Table IV). The first seven amino acids, HVPGGGS, are from the insert in the tubulin-binding region of 4-repeat tau. The corresponding sequence in 3-repeat tau, HQPGGGK, was also detected in this fraction. No phosphorylated residues were detected on any of the digestion products from this F5.5 sample.

The second F5.5 preparation was digested with trypsin. The digest was separated into eight fractions by rp-HPLC (Fig. 6). Mass spectrometric analyses and Edman sequencing of these fractions resulted in the identification of peptides that covered 70% of the 93-amino acid region of 4-repeat tau described above (Table IV). Fractions 6 and 7 of the trypsin
Phosphorylation Sites on Normal and PHF-Tau

The peptides identified in both the asp-N and trypsin digests provided 100% coverage of the 93-amino acid region. Amino acids 299–391 of human r+441 is 100% homologous with amino acids 288–380 of bovine tau, and 81% of this sequence was covered in the analysis of bovine tau and no phosphate was detected. For porcine tau, 97% of the corresponding sequence (241–333 of r+383) was recovered, which was also 100% homologous with human tau and contained no phosphate.

Together these F5.5 data indicate that no phosphorylation sites were detected within the 93-amino acid region identified in the F5.5 fraction. Therefore phosphate-dependent antibodies to PHF-tau must react with epitopes outside of this 93-amino acid region of F5.5 tau. Jakes et al. (1991) have previously Edman sequenced F5.5 and identified three fragments. One fragment corresponded to the end of repeat one plus repeats 3 and 4 from 3-repeat tau. The other fragments were derived from 4-repeat tau and corresponded to the end of repeat 2 plus repeats 3 and 4, and the end of repeat one plus repeats 2 and 3. This study confirmed the presence of the first two fragments. The third contains the amino acids 275–298 of r+441. We did not find any evidence of this sequence. In addition two carboxyl termini were identified, corresponding to E380 and E391 of human r+441, which were not predicted based on asp-N cleavage. The presence of ubiquitin in the F5.5 sample that was not affinity-purified confirms previous results from antibody studies (Cole and Timiras, 1987; Grundke-Iqbal et al., 1988) in which ubiquitin was inferred to be associated with PHFs.

Immunoreactivities of Bovine and Porcine tau Proteins—The same bovine and porcine tau protein preparations that were analyzed by mass spectrometry were also probed with antibodies whose immunoreactivities are known to be dependent on the phosphorylation state of tau. First bovine and porcine tau proteins were treated with alkaline phosphatase and electrophoresed to assess whether proteases that might contaminate the phosphatase preparation would degrade tau protein. Slight increases in the mobilities of the various tau bands were observed after incubating with alkaline phosphatase, consistent with dephosphorylation, but no major shifts indicative of proteolysis were observed (Fig. 7A).

Porcine tau proteins, with and without alkaline phosphatase treatment, were assessed for reactivity with antibody T3P. T3P is an antiserum produced against peptide T3 (GAEIVYKSPVVSGD) that was phosphorylated on the serine residue within the KSPV motif (S396 of r+441; Lee et al., 1991). Although this antiserum may detect more than one epitope on peptide T3, it is selective for the phosphorylated form of the peptide (Lee et al., 1991). Substantial T3P reactivity was detected on Western blots of porcine tau (Fig. 7B), even though no phosphate was chemically detected within its proposed epitope (see mass spectrometric data). T3P reactivity was abolished upon alkaline phosphatase treatment, indicating that the T3P immunoreactivity was dependent on the presence of phosphate.

ELISA was used to assess further the reactivities of porcine and bovine tau proteins with several PHF-relevant antibodies. Bovine tau was reactive with T3P by ELISA, and both tau proteins were reactive with monoclonal antibody SMI34 (Figs. 8 and 9). These reactivities were lost upon alkaline phosphatase treatment of tau, indicating that both antibodies reacted with phosphorylated sites on both proteins. Alkaline phosphatase from E. coli was more effective than alkaline phos-
Phosphorylation Sites on Normal and PHF-Tau

**FIG. 4.** Electrospray mass spectrum showing the phosphorylation state of the peptide equivalent to amino acids 193–251 of human \(\tau_{441}\). The mass spectrum was measured on a VG Bio-Q mass spectrometer. The spectrum shows the presence of unphosphorylated peptide (Series A; \(M_r = 6035.1 + 0.6\) a.m.u.), monophosphorylated peptide (Series B; \(M_r = 6115.0 + 0.2\) a.m.u.), and diphosphorylated peptide (Series C; \(M_r = 6194.7 + 0.5\) a.m.u.).

**TABLE II**

Peptides identified from an Asp-N digest of porcine Taus by mass spectrometry and Edman sequencing

Fraction numbers refer to the elution position on rp-HPLC. The numbers in parentheses refer to the amino acid positions in human \(\tau_{441}\).

| Fraction | m/z    | Predicted peptide                           |
|----------|--------|---------------------------------------------|
| 1        | 688.3  | DTPNLE (110–115)                            |
|          |        | DQAAGHVTQARMVSKGKDGTG-PDDKTKTG (116–144)*  |
| 2        | 1289.8 | DHAHTGYGLGDRK (13–24)                       |
| 3        | 1658.9 | DTSPRHLSNVSTGSI (402–417)                   |
|          |        | DTSPRHLSNVSTGSI (402–417 + PO₄)             |
| 5        | 1102.6 | DREVQSKIGSL (348–357)                       |
| 6        | 915.5  | DSPQLATLA (421–429)                         |
|          | 1557.9 | DHGAEIVYKPSVSVG (387–401)                   |
| 7        | 3206.4 | DNIITHVPGGNKKIETHKLTFT-RENAKAFT (358–386)  |
| 8        | 1217.6 | DAEVSALAKQGL (430–441)                      |
| 9        | 2463.6 | DTSPRHGGNKKIETHKLTFT (338–379)              |
| 10       | 2004.1 | DTSPHLNSVTGSI (402–420)                     |
|          | 2083.3 | DTSPHLNSVTGSI (402–420 + PO₄)               |
| 11       | 2008.2 | DNIKHVPGGSVQIVYKPV (295–313)                |
|          | 3234.1 | DLSKVTSKCSFLNQHHPGG-GQVEKSEKL (314–344)     |
|          | 3234.7 | DKLKVRSKIGSTENLKHQPQ-GKVQINJKL (252–282)*   |

*Peptides assigned by Edman sequencing only.

The results of the present study indicate that both bovine and porcine tau proteins are phosphorylated at the serine homologous to human S404. This residue can be phosphorylated in vitro by tau protein kinase (Ishiguro et al., 1991) and mitogen-activated protein kinase (Gustke et al., 1992). Since these tau proteins were not assembled into PHFs, phosphorylation at S404 is not by itself adequate for tau to form into PHFs. A study using rat tau suggested this residue is phosphorylated in a developmentally regulated fashion (Kanemaru et al., 1992).

**DISCUSSION**

The results of the present study indicate that both bovine and porcine tau proteins are phosphorylated at the serine homologous to human S404. This residue can be phosphorylated in vitro by tau protein kinase (Ishiguro et al., 1991) and mitogen-activated protein kinase (Gustke et al., 1992). Since these tau proteins were not assembled into PHFs, phosphorylation at S404 is not by itself adequate for tau to form into PHFs. A study using rat tau suggested this residue is phosphorylated in a developmentally regulated fashion (Kanemaru et al., 1992).

The KSPV sequence was not phosphorylated on bovine or porcine tau. Therefore antibodies T3P and SM34 could not have reacted with the phosphorylated KSPV site, their assumed epitope, even though both antibodies were shown to
**TABLE III**

Peptides identified from an Asp-N digest of F5.5 extract

The peptides were assigned to human tau sequences on the basis of mass spectrometry and Edman sequencing. Fraction numbers refer to the elution position on rp-HPLC (Fig. 5). The numbers in parentheses refer to the amino acid positions within the fragment of 4-repeat human tau shown below, homologous to amino acids 299–391 of human r441. Fraction 6 was assigned by Edman sequencing only.

| Fraction | Observed m/z | Theoretical m/z | Predicted Peptide |
|----------|--------------|-----------------|-------------------|
| 1        | 1102.6       | 1102.6          | DRVQSKIGSL (50-59) |
| 2        | 1103.7       | 1103.7          | DRVESKIGSL (50-59) |
| 3        | 3716.6 +/- 0.64* | 3716.1 | DNTIVPGGNKIKETHKLTPRAKATFDGAE (69-93) |
| 4        | 2592.6       | 2592.9          | DNTIVPGGNKIKETHKLTPRAKATFDGAE (69-93) |
| 5        | 4800.8 +/- 0.69* | 4800.3 | DRVQSKIGSLDNITVPGGNKKIKETHKLTPRAKATFDGAE (50-93) |
| 6        | 3676.8       | 3677.2          | DRVQSKIGSLDNITVPGGNKKIKETHKLTPRAKATFDGAE (50-82) |
| 7        | 2592.6       | 2592.9          | HVPGGGSVQIVKVDVL-SK (1-19) |

*Peptides assigned using Electrospray mass spectrometry.

**TABLE IV**

Peptides identified from a trypsin digest of F5.5 extract

The peptides were assigned to human tau sequences on the basis of mass spectrometry and Edman sequencing. Fraction numbers refer to the elution position on rp-HPLC (Fig. 6). The numbers in parentheses refer to the amino acid positions within the fragment of 4-repeat human tau shown below, homologous to amino acids 299–391 of human r441. Fraction 8 contained intact ubiquitin (Schlesinger and Goldstein, 1975).

| Fraction | Observed m/z | Theoretical m/z | Predicted Peptide |
|----------|--------------|-----------------|-------------------|
| 1        | 521.8        | 522.3           | LDFK (46-49)      |
| 2        | 535.9        | 536.3           | LTFR (78-81)      |
| 3        | 866.1        | 866.5           | SKLDFK (43-49)    |
| 4        | 1917.2       | 1917.0          | COSLONKIKPGGOQVEVK (24-42) |
| 5        | 1578.8       | 1578.8          | IGLONKTHPGGGNKK (56-71) |
| 6        | 1388.4       | 1388.8          | VQIVKVVPDLSK (8-19) |
| 7        | 1980.4       | 1980.1          | HVGQGQSYQIVKVPDL-SK (1-19) |
| 8        | 8451.7 +/- 1.7* | 8451.8 | Ubiquitin |

*Peptides assigned using Electrospray mass spectrometry.

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**Fig. 6. Peptide map of F5.5 extract after digestion with trypsin.** 100 μg of F5.5 was digested with trypsin, and the resulting digest was separated by rp-HPLC as described under “Materials and Methods.” Numbers indicate fractions that were analyzed by mass spectrometry and Edman sequencing. The phosphates were present on a bovine tau peptide that included the tau-1 epitope, these phosphates did not alter tau-1 reactivity and therefore are not likely to be homologous to those on PHF-tau. (The same phosphates may be present on porcine phosphates were present on a bovine tau peptide that included the tau-1 epitope, these phosphates did not alter tau-1 reactivity and therefore are not likely to be homologous to those on PHF-tau. The same phosphates may be present on porcine

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**Fig. 7. Western blot analyses of tau proteins.** A, 1.6 μg of bovine tau alone or after treatment with E. coli alkaline phosphatase were analyzed with antibody tau-14 at a 1:5000 dilution. B, 4.0 μg of porcine tau alone (−) or after E. coli alkaline phosphatase treatment (+) was analyzed with antibody T3P at a 1:100 dilution. Note that the bovine tau bands are displaced by alkaline phosphatase in the + lane in A.
tau but the homologous peptide was not observed by mass spectrometric analysis.) These results leave open the possibility that a phosphate on tau that alters tau-1 reactivity is specific for PHF-tau. However, recent studies of human tau expressed in cell cultures suggests that the tau-1 epitope can also be occluded on non-PHF tau.

Recently, Hasegawa et al. (1992) published the first direct analysis of normal and PHF-tau by mass spectrometry and enzyme sequencing. They found amino acids 396–438 of normal human +411 to contain one phosphate and to exist in the phosphorylated and nonphosphorylated forms. We have refined the assignment further to S404 of human +411 (by analogy to porcine and bovine tau) and have shown the degree of phosphorylation to be variable. It has also been shown (Hasegawa et al. 1992) that T231, S235, and S262 are phosphorilayation sites specific to PHF-tau and that there is also a phosphorylation site in the tau-1 region, amino acids 191–225. All sites (except S235 which was not covered by mass spectrometric mapping of normal tau) were shown not to be phosphorylated in normal human tau. Our data show that amino acids 193–251 of normal human +411 (by homology with bovine and porcine tau proteins) contains two phosphorylation sites, which would agree with the data of Hasegawa et al. (1992) providing both phosphorylation sites lie within amino acids 235–251 of human +411. (This region of normal tau was not covered by their experiments.)

No phosphorylation sites or other post-translational modifications were identified in the tubulin-binding region of PHF-tau (F5.5) in the present study. Our data agrees with and extends the data of Hasegawa et al. (1992) for this region of PHF-tau. This region of tau seems very important for several reasons. Unlike its binding to microtubules, binding of this region to the rest of the PHF structure is very strong, which renders this region of tau resistant to pronase treatment. The present results show that this binding is not due to phosphorylation, suggesting that it may be a property of non-PHF tau as well.

The unusual binding capacity of F5.5 tau may be integral to PHF formation. This almost irreversible binding of F5.5 tau to PHFs may be detrimental to cells, because it may sequester tau in PHFs, rendering it unavailable for its normal cellular functions. This region of tau, but not full length tau, has been shown to assemble into PHF-like fibrils (Wille et al., 1992). This region can also induce polymerization of the carboxy terminus of β-amyloid precursor protein into PHF-like fibrils (Caputo et al., 1992a).

The absence of post-translational modifications on F5.5 tau indicates that the reactivities of two antibodies specific for F5.5 tau, 6.423 and 728 (Wischik et al., 1988; Caputo et al.,

\[ \begin{align*} 
& & \text{FIG. 8. Immunoreactivity of porcine tau protein with several phosphate-relevant tau antibodies.} 
& & \text{Porcine tau alone (hatched bars) or treated with E. coli alkaline phosphatase (cross-hatched bars) or calf intestine alkaline phosphatase (open bars) was analyzed by ELISA with the antibodies at the dilutions indicated. Color development times were 20 min for SMI34 and 10 min for tau-1, * absorbance was equal to or below that of control wells with no tau present.} 
\end{align*} \]

\[ \begin{align*} 
\text{FIG. 9. Immunoreactivity of bovine tau protein with several phosphate-relevant tau antibodies.} 
\text{Bovine tau (0.05 µg) alone (hatched bars) or treated with E. coli alkaline phosphatase (cross-hatched bars) was analyzed by ELISA with the antibodies at the dilutions indicated. Color development times were 60 min for T3P, 15 min for SMI34, and 7.5 min for tau-1. * absorbance was equal to or below that of control wells with no tau present. Means ± S.E. are shown.} 
\end{align*} \]
Phosphorylation at a site outside of the tubulin-binding region of tau may be sufficient to either induce tau assembly into PHFs or to release tau from microtubules, where it may be more susceptible to polymerization. These sites will have been identified by direct chemical analysis of intact tau from a single PHF-unique site. Instead the total number of phosphorylation sites on non-PHF tau proteins that were phosphorylated in vivo and are not candidates for inducing PHF formation. This study also demonstrates that a 93-amino acid segment of the tubulin-binding region of PHF-tau is not phosphorylated and that ubiquitin is present in the preparation. Finally, several Alzheimer-related antibodies were shown to react with non-Alzheimer tau in a phosphate-dependent manner, although probably not at the epitopes they were originally thought to recognize.

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