Proline-directed Phosphorylation of Human Tau Protein*

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The primary sequence of the microtubule-associated protein tau contains multiple repeats of the sequence -X-Ser/Thr-Pro-X-, the consensus sequence for the proline-directed protein kinase (p34<sup>cdc2</sup>/p58<sup>yck</sup> A). When phosphorylated by proline-directed protein kinase in vitro, tau was found to be a “hyperphosphorylated” form of tau (phosphorylated to a stoichiometry of greater than 1). The kinase(s) responsible for this hyperphosphorylation, the phosphorylation sites which constitute the hyperphosphorylated state, and the role of this hyperphosphorylation in the formation of neurofibrillary tangles are unknown. Evidence exists that a serine residue equivalent to Ser-307 is abnormally phosphorylated in AD (19, 20). However, it is postulated that other sites remain to be identified. The primary amino acid sequence of tau contains 14 potential sites for the proline-directed protein kinase (PDPK), a recently discovered growth factor-activated protein kinase identified to be a heterodimer of p58<sup>yck</sup> A/p34<sup>cdc2</sup>. This protein kinase recognizes the minimal consensus sequence of -X-(Ser/Thr)-Pro-X- (22-24). We report here that PDPK hyperphosphorylates tau and identify the phosphorylated sites. The relevance of this phosphorylation to AD is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—N-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (EC 3.4.21.4) was from Worthington Biochemical Corp.; [γ-<sup>32</sup>P]ATP was from ICN; cellulose plates for thin layer chromatography were from Kodak. The Tau-1 antibody was the generous gift of Dr. Lester Binder, University of Alabama at Birmingham.

**Purification of Tau**—Tau was purified from extracts of Escherichia coli.
coli BL21 (DE 3) LysS cells transformed with pET-3d plasmid (25) harboring the human three-repeat tau cDNA. The cells were lysed in 30 mM Tris, pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 1 mM dithiothreitol, 2.5 μg/ml DNase I, and centrifuged at 23,400 × g for 20 min. The supernatant was applied to diethylaminoethyl cellulose (Whatman DE-52) and the flow-through collected and chromatographed on phosphocellulose (Whatman P-11) in 50 mM potassium Pipes buffer, 1 mM EGTA, 0.2 mM MgCl₂, pH 6.8. The tau protein was eluted in the same buffer with a gradient of 0.2–0.4 M KCl. After dialysis, the tau protein was concentrated in an Amicon micro concentrator.

Purification of Proline-directed Protein Kinase—The proline-directed kinase was purified from mouse FM3A cells by a slight modification of the method of Hall et al. (24). Modifications include: isolation from FM3A cells maintained in spinner flasks in logarithmic growth in HEPES-buffered RPMI-1640 medium supplemented with 10% calf serum and chromatography on Q-Sepharose FF (Fast Flow, Pharmacia Fine Chemicals) instead of DEAE-cellulose. The column was washed with equilibration buffer containing 10 μM cyclic AMP and then equilibration buffer containing 150 mM NaCl. PDPK activity was eluted from Fast Q with equilibration buffer containing 350 mM NaCl. Throughout the various purification steps, the kinase activity was assayed using a highly selective synthetic peptide substrate derived from tyrosine kinase substrate (TKS) (26), (27) as described by Vallat et al. (22). PDPK prepared by this method had a specific activity of 5–20 units/μg protein (1 unit of activity is defined as the amount of kinase necessary to transfer 1 pmol of phosphate/min from ATP to TH₂⁻⁻ peptide when assayed under standard assay conditions: 100 mM Tris-acetate, pH 7.6, 100 μM peptide substrate, 100 μM ATP, and 10 mM magnesium acetate.

Phosphorylation of Tau with PDPK and Phosphopeptide Mapping—Typically 2.5 μg of purified human recombinant tau protein was phosphorylated by PDPK under standard assay conditions using 3–5 units of PDPK and incubation for 60 min at 30 °C in a final volume of 50 μl. The reaction was stopped by addition of 5.5 μl of 70% perchloric acid. Following centrifugation for 5 min at 13,000 × g, the pellets were washed three times with 1 ml of 25% trichloroacetic acid, twice with 1 ml of cold acetone, and the incorporated phosphate estimated by Cerenkov counting. The acid precipitate was then resuspended in 25 μl of SDS sample buffer and separated on 10% SDS-polyacrylamide gels by the method of Laemmli (26).

For phosphopeptide mapping, the pellets were resuspended in 100 μl of 0.1 M N-morpholine acetate, pH 8.3, and trypsin added at a ratio of 1:10 trypsin:protein (w/w). Following digestion at 37 °C overnight, the samples were analyzed on a Gilson gradient high performance liquid chromatograph equipped with a 0.46 × 25-cm Vydac C₁₈ column at 8°C (Fig. 2A). The reaction was stopped by addition of 5.5 μl of 10% acetic acid, twice with 1 ml of cold acetone, and the incorporated phosphate estimated by Cerenkov counting. The acid precipitate was then resuspended in 25 μl of SDS sample buffer and separated on 10% SDS-polyacrylamide gels by the method of Laemmli (26).

Purified recombinant human tau was incubated in the presence of PDPK (22–24) and then analyzed on SDS-polyacrylamide gel (Fig. 1A). Use of recombinant tau in these studies circumvents the intrinsic heterogeneity of brain protein and also provides a non-phosphorylated substrate for this protein kinase. The stained gel (Fig. 1A) shows that tau incubated in the absence of PDPK (Fig. 1A, lane 1) has a slightly greater mobility than tau incubated in the presence of the protein kinase (Fig. 1A, lane 2). This result is consistent with previous reports demonstrating a decreased mobility of phosphorylated forms of tau (15, 19–21). The autoradiogram of the stained gel (Fig. 1B) confirms that 32P is incorporated into tau only in the presence of the protein kinase. PDPK was found to transfer 4.4 mol of 32P-labeled phosphate/mol of tau protein under conditions in which this kinase would transfer 0.9 mol of phosphate/mol into synapsin (24).

The ability of PDPK to phosphorylate tau to a stoichiometry greater than one suggested that this protein might contain more than one proline-directed phosphorylation site. To examine this possibility, tau was incubated in the presence of PDPK and [γ-32P]ATP and then digested with trypsin. The analysis of the tryptic phosphopeptides by isoelectric focusing (IEF) revealed five distinct radioactive bands (labeled A–E) with respective isoelectric points of 6.9, 6.5, 5.6–5.9, 4.7, and 3.6 (Fig. 2A). The percentage of the total incorporated radioactivity in each phosphopeptide was as follows: A, 40%; B, 26%; C, 7%; D, 22%; E, 5%. HPLC analysis of a similar tryptic digest showed the presence of only three major peaks of radioactivity (Fig. 2B). When each IEF band was isolated and analyzed by the HPLC, the chromatogram revealed only a single peak of radioactivity, suggesting that each IEF band contained a single phosphopeptide (data not shown).

The retention times of the individual IEF phosphopeptides are indicated in the HPLC chromatogram with the labeled arrows (Fig. 2B). Tryptic phosphopeptide pair A and C and pair D and E do not resolve under these chromatographic conditions.

**RESULTS AND DISCUSSION**

Purified recombinant human tau was incubated in the presence of PDPK (22–24) and then analyzed on SDS-polyacrylamide gel (Fig. 1A). Use of recombinant tau in these studies circumvents the intrinsic heterogeneity of brain protein and also provides a non-phosphorylated substrate for this protein kinase. The stained gel (Fig. 1A) shows that tau incubated in the absence of PDPK (Fig. 1A, lane 1) has a slightly greater mobility than tau incubated in the presence of the protein kinase (Fig. 1A, lane 2). This result is consistent with previous reports demonstrating a decreased mobility of phosphorylated forms of tau (15, 19–21). The autoradiogram of the stained gel (Fig. 1B) confirms that 32P is incorporated into tau only in the presence of the protein kinase. PDPK was found to transfer 4.4 mol of 32P-labeled phosphate/mol of tau protein under conditions in which this kinase would transfer 0.9 mol of phosphate/mol into synapsin (24).

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**FIG. 1. SDS-polyacrylamide gel electrophoresis and autoradiographic analysis of human tau phosphorylated by the proline-directed protein kinase.** Purified recombinant human tau was incubated at a concentration of 1 μM in the presence or absence of PDPK (22) as described under "Experimental Procedures." The samples were then suspended in SDS sample buffer and analyzed on 10% SDS-polyacrylamide gel (26). The gel was stained with Coomassie Brilliant Blue R, destained, dried, and analyzed by autoradiography. A, the Coomassie-stained gel: lane 1, tau alone; lane 2, tau + PDPK; lane 3, PDPK alone. B, Autoradiograph of gel shown in A.
Phosphoamino acid standards is indicated by the phosphoserine; PT, phosphothreonine; proline-directed protein kinase as described under "Experimental Procedures." Following digestion with trypsin at 37 °C overnight, samples were analyzed by isoelectric focusing (A) or HPLC (B). A, tau phosphopeptides were analyzed on isoelectric focusing gels (Serva Precote gels, pH 3-10) by the manufacturer’s directions, and the radioactive peptides were identified by autoradiography. The PDPK phosphorylated tau revealed the presence of five peptides by isoelectric focusing. B, tau tryptic phosphopeptides were analyzed on a high performance liquid chromatograph. To establish the relationship between the peptides isolated by each technique, phosphopeptides were extracted from the IEF gel and individually analyzed on HPLC. The designated arrows indicate the elution position of each of the individual phosphopeptides. C, phosphoamino acid analysis of the tryptic phosphopeptides isolated in A, the position of authentic phosphoamino acid standards is indicated by the arrows. PS, phosphoserine; PT, phosphothreonine; PY, phosphoryrosine.

The phosphopeptides were also subjected to phosphoamino acid analysis (27). Peptide B demonstrated the presence of phosphoserine, while peptides A, D, and E contained both phosphoserine and phosphothreonine (Fig. 2C).

Since the amino acid sequence of human tau is known (18, 30), Edman sequencing of these phosphopeptides was employed to locate the phosphorylation sites within the tau protein. Table I lists the results from gas phase sequence analysis of purified peptides A, B, and D. A single phenylthiohydantoin-derivative (PTH-derivative) at each cycle during sequencing of the peptide confirmed that only one peptide species was present. Although peptide D could not be sequenced to completion, the remaining amino acids can be deduced from the amino acid composition determined for each of the peptides (data not shown). Definitive sequence data could not be obtained from peptide C or peptide E.

Comparisons of the peptide sequences to the human tau sequence as predicted from cDNA showed that peptide A corresponded to residues 167-182 and peptide B corresponded to residues 173-182 (18, 30). Since phosphoamino acid analysis demonstrated that peptide A contained both phosphothreonine and phosphoserine (Fig. 2C), threonine 173 must be phosphorylated as inspection of the sequence reveals only 1 threonine in this peptide. Peptide B, which lacks a phosphothreonine on phosphoamino acid analysis and exhibits a PTH-Thr equivalent to threonine 173 on gas phase sequencing, contains threonine 173 in the non-phosphorylated state. The phosphate present on Thr-173 in peptide A would be expected to render tau protein resistant to tryptic cleavage at Arg-172 (31). Consequently, peptide B most likely results from incomplete phosphorylation of tau at Thr-173 allowing trypsinolysis at Arg-172 to yield a peptide containing residues 173-182.

Protein sequencing by Edman degradation depends on the quantitative recovery of the PTH-derivative at each cycle. However, when a phosphorylated serine or threonine residue is encountered during Edman sequencing, little or no PTH-derivative is recovered at that cycle. The low recovery of an expected serine or threonine derivative at a particular cycle followed by adequate recoveries of the next amino acid in subsequent cycles of degradation is characteristic of a phosphorylated residue. On this basis, the data in Table I reporting the recovery of the amino acid derivatives independently suggest that Thr-173 in peptide A is phosphorylated. Similarly, the sequencing data for peptide B suggest that of the 3 serines in this peptide, only Ser-177 is phosphorylated.

The protein sequencing and amino acid composition data indicate that Peptide D corresponds to tau residues 137-151 (18, 30). As it contained both phosphoserine and phosphothreonine, Thr-147 can be identified as a phosphorylation site since it is the only threonine in the peptide. The identity of the phosphorylated serine in peptide D is more difficult from

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**TABLE I**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Peptide A</th>
<th>Peptide B</th>
<th>Peptide D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residue</td>
<td>Amount</td>
<td>Residue</td>
</tr>
<tr>
<td>1</td>
<td>Lys</td>
<td>72</td>
<td>Thr</td>
</tr>
<tr>
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<td>Val</td>
<td>80</td>
<td>Pro</td>
</tr>
<tr>
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<td>Ala</td>
<td>85</td>
<td>Pro</td>
</tr>
<tr>
<td>4</td>
<td>Val</td>
<td>75</td>
<td>Lys</td>
</tr>
<tr>
<td>5</td>
<td>Val</td>
<td>71</td>
<td>(Ser)</td>
</tr>
<tr>
<td>6</td>
<td>Arg</td>
<td>33</td>
<td>Pro</td>
</tr>
<tr>
<td>7</td>
<td>(Thr)</td>
<td></td>
<td>Ser</td>
</tr>
<tr>
<td>8</td>
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<td>9</td>
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</tr>
<tr>
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</tr>
<tr>
<td>11</td>
<td>(Ser)</td>
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</tr>
<tr>
<td>12</td>
<td>Pro</td>
<td>7</td>
<td></td>
</tr>
<tr>
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<td>Ser</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>15</td>
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</tr>
<tr>
<td>16</td>
<td>(Lys)</td>
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</table>
When the data from gas phase sequencing, amino acid analysis, and phosphoamino acid analysis are taken together, the identity of the serine residue can be deduced. Since PDPK requires the -X-(Ser/Thr)-Pro-X- motif (22-24) and the three phosphorylated residues identified thus far all possess this motif, the modified serine residue in peptide D most likely would have the same motif. Only 2 of the 5 serines in peptide D fit this criteria, Ser-141 and Ser-144. From Table I, the recovery of PTH-derivatives from cycles corresponding to the serines at positions 137, 140, and 141 suggest that these serines are not phosphorylated. Given that no other protein kinase activities have been detected in this preparation of PDPK and that no other peptides co-purify with peptide D, it can be inferred that Ser-144 is the phosphorylated serine residue since no PTH-AA is detected at the cycle corresponding to Ser-144 and its location is in the requisite motif for proline-directed phosphorylation. Although the data supporting this conclusion is indirect, serine 144 is the most probable serine residue phosphorylated by PDPK in peptide D.

The phosphopeptides sequenced accounted for 87% of the total radioactive recovery from the isoelectric focusing step, indicating that the major PDPK phosphorylation sites were identified. The remaining sites are not likely to have been phosphorylated with high efficiency. Interestingly, not all of the fourteen potential proline-directed phosphorylation sites (i.e. the -X-(S/T)-P-X- motifs in tau are phosphorylated by PDPK, indicating that this motif is a necessary but not sufficient "discriminator" for this protein kinase. Comparison of the adjacent residues on both sides of all actual and potential PDPK phosphorylation sites does not indicate any pattern in the primary structure other than the known requirement of a carboxyl-side proline (22-24); the answer awaits elucidation of tau's tertiary structure to determine accessibility of potential phosphorylation sites to the protein kinase.

Two of the four identified phosphorylation sites (Ser-144 and Thr-147) are located within the region of tau identified as the epitope for the monoclonal antibody Tau-1 (residues 131-149) (32). Fig. 3 shows a comparison of their relative mobility and cross-reactivity to the Tau-1 monoclonal antibody. Tau and control monoclonal antibody, failed to detect any difference in reactivity between the two lanes (data not shown).

The identification of Ser-144 and Thr-147 as residue(s) that affect Tau-1 reactivity is relevant to the tau protein in neurofibrillary tangles of AD. The first indication that tau in AD was abnormally phosphorylated followed from the observation that phosphatase treatment of AD brain sections was necessary for Tau-1 reactivity (34, 35). It was also noted that phosphatase treatment for Tau-1 reactivity (19-21). The classification of these phosphorylated sites as abnormal would be premature, since phosphatase-dependent Tau-1 reactivity has also been described in normal adult rat brain tissue (36). However, an increase in phosphatase-dependent Tau-1 reactivity has been reported in AD brain extracts, which may reflect axonal disruption and the dislocation of tau from the axon into the somatodendritic region (37). Therefore, it can be argued that in AD, Ser-144 and Thr-147 are abnormally phosphorylated in the sense that they are phosphorylated to a higher stoichiometry than normal.

Proline-directed protein kinase is known to phosphorylate other neuronal structural proteins including synapsin I (24), neurofilament (38), and MAP-2 (39). The phosphorylation sites Thr-147, Thr-173, and Ser-177 are conserved relative to the repeat domain of tau which constitutes the microtubule binding sites, Thr-173 corresponds to a conservatively substituted threonine in MAP-2 (40). These sites lie in two small regions of homology between MAP-2 and tau, surrounded by stretches of non-homology (Fig. 4). While none of the sites are in the repeat domain of tau which constitutes the microtubule binding sites, Thr-173 and Ser-177 lie in an area that enhances the binding of tau to microtubules in vitro and in vivo (41). Although phosphorylation has been reported to affect tau and MAP-2 activity (14, 43), functional effects mediated by PDPK phosphorylation of human tau protein is schematized to indicate the relative positions of the microtubule-binding repeat domains (gray boxes) to the region of tau phosphorylated by PDPK (hatched lines). The phosphopeptides isolated and sequenced from tau (residues 131-197) are boxed and their relative position within the molecule indicated on the exploded diagram. The phosphorylation sites are illustrated in boldface type. The known epitope for the binding of Tau-1 monoclonal (residues 131-149) is identified with a bar above the minimally necessary sequence. The homologous region of microtubule associated protein-2 is positioned in the same manner as tau with respect to the repeat microtubule-binding domains. Identical residues are paired with a solid line, while conservative differences are paired with broken lines.
ation remain to be shown. None of the PDKP phosphorylation sites in tau and MAP-2 are conserved in the non-neuronal MAP-4 sequence, although this protein contains a microtubule-binding repeat domain homologous to that of tau and MAP-2 (44, 45).

It is very likely that other proline-directed protein kinases, in addition to PDKP, will be able to hyperphosphorylate tau. The relationship of PDKP to two tau protein kinases recently isolated from bovine brain is unknown (46-48). Tau protein kinase II, which appears to require an adjacent proline on the carboxyl terminus of the phosphorylated residue was identified to phosphorylate Ser-144, Thr-147, Ser-177, and Ser-315 in bovine brain tau (47). The similarity of the phosphorylation sites in tau modified by both of these protein kinases suggest that they are closely related. Tau protein kinase II was noted to be activated by the presence of tubulin in the assay (46, 48), whereas PDPK is activated by treatment of PC12 cells with nerve growth factor (22) and PDKP is activated by the presence of tubulin in the assay (46, 48), whereas PDPK is activated by treatment of PC12 cells with tubulin-binding repeat domain homologous to that of tau and MAP-2 (44, 45).


tau protein and do not phosphorylate casein (23, 48). The specific phosphate-dependent epitope for Tau-1 will further investigations of the biochemical mechanisms controlling these disease processes.

**Acknowledgment**—We sincerely thank Dr. Roland Brandt for supplying the human tau protein.

**REFERENCES**


S. M. Halloran and R. Vulliet, unpublished observation.