Alzheimer Disease-specific Conformation of Hyperphosphorylated Paired Helical Filament-Tau Is Polyubiquitinated through Lys-48, Lys-11, and Lys-6 Ubiquitin Conjugation*

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One of the key pathological hallmarks of Alzheimer disease (AD) is the accumulation of paired helical filaments (PHFs) of hyperphosphorylated microtubule-associated protein Tau. Tandem mass spectrometry was employed to examine PHF-Tau post-translational modifications, in particular protein phosphorylation and ubiquitination, to shed light on their role in the early stages of Alzheimer disease. PHF-Tau from Alzheimer disease brain was affinity-purified by MC1 monoclonal antibody to isolate a soluble fraction of PHF-Tau in a conformation unique to human AD brain. A large number of phosphorylation sites were identified by employing a data-dependent neutral loss algorithm to trigger MS3 scans of phosphopeptides. It was found that soluble PHF-Tau is ubiquitinated at its microtubule-binding domain at residues Lys-254, Lys-311, and Lys-353, suggesting that ubiquitination of PHF-Tau may be an earlier pathological event than previously thought and that ubiquitination could play a regulatory role in modulating the integrity of microtubules during the course of AD. Tandem mass spectrometry data for ubiquitin itself indicate that PHF-Tau is modified by three polyubiquitin linkages, at Lys-6, Lys-11, and Lys-48. Relative quantitative analysis indicates that Lys-48-linked polyubiquitination is the primary form of polyubiquitination with a minor portion of ubiquitin linked at Lys-6 and Lys-11. Because modification by Lys-48-linked polyubiquitin chains is known to serve as the primary path to target proteins for degradation by the ubiquitin-proteasome system, and it has been reported that modification at Lys-6 inhibits ubiquitin-dependent protein degradation, a failure of the ubiquitin-proteasome system could play a regulatory role in modulating the integrity of microtubules and interaction with cytoskeletal components.

The major pathological hallmarks of Alzheimer disease (AD) are the extracellular formation of senile plaques composed of the amyloid β peptide and the intraneuronal formation of neurofibrillary tangles (NFTs), which have a degradation-resistant core made up of paired helical filaments (PHFs) of the microtubule-associated protein Tau (PHF-Tau). In the AD brain, the degree of NFT formation has been shown to correlate more closely to the loss of neuronal function than the degree of senile plaque accumulation (1). A recent study by SantaCruz et al. (2) has shown, however, that the existence of NFTs alone does not cause neuronal death, implying that a pre-tangle form of Tau may be responsible for the neuronal loss and other pathological symptoms characteristic of disorders involving the Tau protein (tauopathies), including AD.

Tau was initially discovered as a phosphoprotein that promotes assembly of microtubules (3); it was later found that hyperphosphorylated Tau is the major protein comprising the PHFs in AD (4, 5). Because hyperphosphorylated Tau is found in all other tauopathies in addition to AD (6), interest then developed in the role that phosphorylation may play in the formation of PHFs and the development of NFTs.

The stabilization of microtubules is the major known function of Tau. Tau has six isoforms in the adult human brain, which vary in length from 352 to 441 amino acid residues and result from alternative splicing of the tau gene, which is located on human chromosome 17 at 17q21 (7). Tau has an N-terminal projection domain (so named because it projects from the microtubule surface) composed of an acidic region at the N terminus and a proline-rich region in the interior. Various studies have suggested physiological roles of the projection domain, including determining axonal microtubule spacing (8) and interaction or interconnection with cytoskeletal components (9–12), mitochondria (13), and the plasma membrane (14, 15). On its C-terminal side, Tau has a microtubule-binding domain with either three or four microtubule-binding repeat domains, depending on the isoform, and an acidic region at the C terminus. It has been surmised that abnormal phosphorylation events may cause Tau to dissociate from microtubules and form PHFs (16). However, because native Tau is also phosphorylated (17) and in vitro studies demonstrate that Tau need not be phosphorylated to assemble into PHFs (18), the relation between hyperphosphorylation and PHF formation is not clear, much less the relation between Tau hyperphosphorylation and neuronal toxicity (7, 17, 19, 20). Tau in PHFs has also been reported to be ubiquitinated (21). Because polyubiquitinated proteins are targeted for degradation by the ubiquitin-proteasome system (UPS) (22) and studies indicate that PHFs inhibit the UPS (23), the relationship between PHFs and the UPS needs clarification. To understand the role of Tau in neuropathology, there is a need to explicate the temporal relations between Tau phosphorylation, Tau ubiquitination, Tau conformational changes, and PHF forma-
tion and to find out what forms of Tau, during this process, are responsible for disease pathology.

In this study, soluble PHF-Tau was isolated using the conformation-specific antibody MC1, which recognizes only PHF-Tau isolated from AD brain but not Tau isolated from rat, mice, or normal non-AD human brain (24). The affinity-purified PHF-Tau was analyzed by nanospray liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a linear ion trap mass spectrometer to identify post-translational modifications (PTMs), specifically phosphorylation and ubiquitination sites, on both PHF-Tau and proteins associated with PHF-Tau. The use of a data-dependent algorithm to trigger MS3 scans in the event of a neutral loss (NL) corresponding to the presence of a phosphopeptide significantly improved the quality of mass spectra used to identify phosphopeptides and phosphorylated residues.

LC-MS/MS also provides a facile method for clearly identifying ubiquitinated residues. Three ubiquitination sites were found on PHF-Tau (Lys-254, Lys-311, and Lys-353) as well as polyubiquitination at Lys-6, Lys-11, and Lys-48 of ubiquitin itself, a novel finding of this study. Relative quantification by LC-MS/MS was used to determine the extent of polyubiquitination at each of these sites. The results indicate that the Lys-48 linkage is by far the most prominent form of Tau polyubiquitination, indicating that Tau may be targeted for degradation by the UPS, with possible implications for the role of Tau ubiquitination in the accumulation of PHFs in AD. Functional shotgun proteomic analysis identified MC1-immunopurified PHF-Tau as largely associated with cytoskeletal proteins but not with chaperone proteins, suggesting that MC1-immunopurified PHF Tau may represent an early stage of PHF-Tau involved in tangle formation.

A robust set of mass spectrometric and computational methods was employed to identify and quantify PTMs on soluble PHF-Tau without use of site-specific antibodies. Unique findings indicate that PHF-Tau can be modified at its microtubule-binding domain by at least three different types of polyubiquitin conjugation. We hope the results of this study will further efforts to determine the temporal relationships between levels of Tau PTMs and the progression of AD and provide a foundation for the development of better AD diagnostic sets and therapeutic treatments.

**MATERIALS AND METHODS**

**Affinity Purification of PHF-Tau and Enzymatic Digestion**—Tau was affinity-purified from AD brain by MC1 monoclonal antibody as described previously (24).

In solution digestion of affinity-purified PHF-Tau was performed as follows. Aliquots of PHF-Tau containing ~50 µg of protein were digested at 37 °C in 40% methanol, 100 mM NH₄HCO₃, pH 8.5, by three additions of 1% (w/v) trypsin (Promega) or 1% (w/v) Lys-C (Sigma); digestion was performed for periods of 2 h, 2 h, and overnight. The reaction was stopped by the addition of 1% (v/v) acetic acid. The trypsin digest was further purified using a phosphopeptide enrichment kit employing immobilized gallium (Pierce) in order to further isolate phosphopeptides.

In-gel trypsin digestion of affinity-purified PHF-Tau was performed as follows. Following protein visualization via silver staining (Silver Quest silver staining kit, Invitrogen), bands of interest were excised from SDS-polyacrylamide gels and further cut into 1 × 1-mm pieces. Gel pieces were washed with 100 mM NH₄HCO₃, shrunken with aconitnitrile (Burdick and Jackson), and dried in a vacuum centrifuge. Reduction was carried out with 10 mM dithiothreitol (EM) in 100 mM NH₄HCO₃ for 1 h at 60 °C followed by alkylation with 55 mM iodoacetamide (Sigma) in 100 mM NH₄HCO₃ for 45 min at room temperature in the dark. Gel pieces were washed with 100 mM NH₄HCO₃, dried in a vacuum centrifuge. Rehydration with digestion solution (50 µl of H₂O, 50 µl of 100 mM NH₄HCO₃, 5 µl of CaCl₂, and 1.5 µg of trypsin (Promega)) was performed on ice for 45 min. Any remaining supernatant was removed, and 25 µl of digestion buffer (digestion solution without trypsin) was added for overnight enzymatic cleavage at 37 °C. Peptides were extracted at 37 °C for 15 min with shaking once with 50 mM NH₄HCO₃, pH 7.8, and twice with 5% formic acid (EM)/acetonitrile.

**Immunoblot Analysis**—Briefly, 50 µg of protein from an affinity-purified PHF-Tau sample was used for SDS-PAGE analysis. Protein samples were electroblotted onto nitrocellulose membranes, and blots were blocked in 5% nonfat milk, 0.1% Tween 20 in phosphate-buffered saline. Blots were incubated with a monoclonal mouse anti-polyubiquitin antibody (Novus Biologicals) 1:500 overnight at 4 °C. Blots were then incubated in a horseradish peroxidase-conjugated goat anti-mouse (1:20,000) antibody (Pierce) followed by chemiluminescent detection with Super Signal West Pico chemiluminescent substrate (Pierce).

**Mass Spectrometry (MS) and MS/MS, MS3 Data Generation**—MS analysis of PTMs and relative quantification studies were performed using a Thermo Electron LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) with peptides separated by a true nanoflow XtremeSimple LC system (Micro-Teck Scientific, Inc., Vista, CA) equipped with a 150-mm × 75-µm C-18 reverse-phase column (5-µm 300-Å particles) from Micro-Tech Scientific. Peptides were either loaded onto a trap column (Agilent Zorbax C18 guard column or Michrom Bioresearchs peptide cap trap) or loaded directly into the sample loop with 95% solvent A (2% acetonitrile, 0.1% formic acid) and 5% solvent B (95% acetonitrile, 0.1% formic acid) and were then eluted with a linear gradient of 5–25% solvent B for 60 min, and 25–80% solvent B for 10 min. The mass spectrometer was equipped with a nanospray ion source (Thermo Electron) using an uncoated 10-µm inner diameter SilicaTip™ PicoTip™ nanospray emitter (New Objective, Woburn, MA). The spray voltage of the mass spectrometer was 2.0 kV, and the heated capillary temperature was 200 °C. MS/MS and MS3 spectra were acquired using Xcalibur 1.4 software (LTQ). For PTM identification, the five most abundant ions in each MS1 scan were selected for an MS/MS event, subject to dynamic exclusion (ions were excluded for 30 s after being detected twice in 30 s). An MS3 scan was then triggered if, among the three most abundant ions in the MS/MS scan, a neutral loss of 98, 49, or 32.7 Da was detected (corresponding to loss of phosphoric acid on singly, doubly, and triply charged precursor ions). Other mass spectrometric data generation parameters were as follows: collision energy 24%, full scan MS mass range 400–1800 m/z, minimum MS signal 500 counts, minimum MS/MS signal 100 counts, and activation time 120 ms.

Mass spectrometric analysis of proteins that co-immunoprecipitate with PHF-Tau was performed using a Thermo Finnigan LCQ Deca XP mass spectrometer with reverse-phase LC implemented with an Ultra Plus II LC system (Micro-Teck Scientific) using the same type of reverse-phase column as described above. Peptides were loaded onto a Michrom Bioresearchs peptide cap trap at 95% solvent A (2% acetonitrile, 1.0% formic acid) and 5% solvent B (95% acetonitrile, 1.0% formic acid) and then eluted with a linear gradient of 5–60% solvent B for 65 min and 60–90% solvent B for 10 min. Tandem MS/MS spectra were acquired with Xcalibur 1.2 software. A full MS scan was followed by three consecutive MS/MS scans of the top three ion peaks from the preceding full scan. Dynamic exclusion was enabled such that after three occurrences of an ion within 1 min, the ion was placed on the exclusion list for 3 min. Other mass spectrometric data generation
FIGURE 1. Identification of Ser-262 and Thr-231 phosphorylation sites in PHF-Tau by data-dependent neutral loss MS3 algorithm. A, phosphorylation at Ser-262 of Tau. The MS2 spectrum is dominated by a large neutral loss precursor ion of m/z = 529.9 (see inset). The main figure shows the spectrum resulting from an MS3 scan of this neutral loss precursor ion; the spectrum contains all relevant y-ions and all but one b-ion as well as several y- and b-ions. B, phosphorylation at Thr-231 of Tau. The MS2 spectrum likewise contains a large neutral loss precursor ion (m/z = 474.8 (see inset)). (Note: numbering of residues is according to Tau isoform with NCBI accession number NP_005901.)
parameters were as follows: collision energy 35%, full scan MS mass range 400–1800 m/z, minimum MS signal 5 × 10^5 counts, minimum MS/MS signal 5 × 10^3 counts. The mass spectrometer was equipped with a nanospray ion source (Thermo Electron) using an uncoated 10-μm inner diameter SilicaTip™ PicoTip™ nanospray emitter (New Objective, Woburn, MA). The spray voltage of the mass spectrometer was 1.9 kV, and the heated capillary temperature was 180 °C.

Relative Quantification of Polyubiquitination Linkages by Selected Reaction Monitoring (SRM)—To provide a relative quantitative comparison of polyubiquitin chain ligation sites, an instrument method was developed in which SRM was sequentially performed on precursor ions with the empirically determined m/z values of unmodified and modified peptides corresponding to the polyubiquitination sites that had been identified previously in this study by LC-MS/MS analysis. The six relevant peptides, designated Ub-Lys-6-unmodified (1MQIFVK); Ub-Lys-6*-modified (1MQIFVK*TLTGK11); Ub-Lys-11-unmodified (12TITLEVEPSDTIENVK27); Ub-Lys-11*-modified (12TITLEVEPSDTIENVK*27); Ub-Lys-48-unmodified (43LIFAGKQLEDGR54); and Ub-Lys-48*-modified (43LIFAGK*QLEDGR54), were monitored using the following SRM precursor-to-product ion transitions, as listed in Table 4 as follows: Ub-Lys-6-unmodified (MS1, 765.55 to MS2, 506.3); Ub-Lys-6*-modified (MS1, 690.53 to MS2, 1007.7); Ub-Lys-11-unmodified (MS1, 894.87 to MS2, 1002.5); Ub-Lys-11*-modified (MS1, 801.66 to MS2, 1002.5); Ub-Lys-48-unmodified (MS1, 764.04 to MS2, 1115.7); and Ub-Lys-48*-modified (MS1, 731.48 to MS2, 618.2). Scan ranges were determined so as to minimize the duty cycle while maintaining a set of distinctive ions in each of the resulting spectra that would enable the peptide spectra to be distinguished from noise. QualBrowser (Xcalibur) was then used to display only those scans containing distinctive product ions for each spectrum, in order to increase the signal-to-noise ratio. The peaks in the resulting chromatograms were integrated using the ICIS peak detection feature of QualBrowser with 15-point Boxcar smoothing.

Analysis of MS Spectra—A Beta test version of Bioworks (Bioworks 3.1) on a nine node (2-cpu/node) cluster computer from Thermo Electron utilizing the SEQUEST algorithm was used to determine cross-correlation scores between acquired spectra and a human protein database and, in the case of Tau PTM analysis, a database of the six Tau isoforms as well. To identify phosphorylation sites, differential modifications were used of +80 for serine, threonine, and tyrosine and -18 for serine and threonine (corresponding to dehydroalanine and 2-amino-dehydrobutyric acid, respectively). To identify ubiquitinated peptides, a differential modification of +114 was used, corresponding to the double-glycine fragment of ubiquitin that remains conjugated to the lysine residue of the peptide following trypsin digestion (25). Other SEQUEST parameters included threshold 100 (LTQ) or 1000 (LCQ Deca), monoisotopic, enzyme (trypsin or Lys-C), and charge state automatically determined by Bioworks software. For peptide and PTM identification, spectra passing a threshold of cross-correlation versus charge state (1.5 for +1 ions, 2.0 for +2 ions, and 2.5 for +3 ions) were then inspected to verify that all major ions were identified and, in the case of phosphopeptides, that phosphorylated residues identified with a differential modification of -18 were from MS3 scans.

Statistical Validation of Peptide and Protein Assignments following Database Search—The quality of peptide and protein assignments made by SEQUEST was assessed by using the PeptideProphet and ProteinProphet statistical models (as described in Refs. 26 and 27). Briefly, PeptideProphet first applies machine learning language to distinguish correct and incorrect peptide assignments from the data set being analyzed by using an expectation maximization algorithm. The program then computes discriminant scores (F scores) for correct and incorrect peptide assignments using a set of scores generated from SEQUEST search results. Probability scores (pcomp) of peptide assignments for each acquired tandem mass spectrum are then calculated based on the distribution of discriminant (F) scores. These lists of peptide sequences and their respective pcomp scores obtained from PeptideProphet are then used to determine a minimal list of proteins that can explain the observed data and then to compute a probability (Pcomp) that each protein is present in the original complex peptide mixture. This is accomplished utilizing the ProteinProphet program, which also uses a maximum expectation algorithm similar to PeptideProphet. The probability of each protein is then ranked from 0 (incorrect) to 1 (correct). Proteins that are represented by numerous peptides, high percentage sequence coverage, or extremely strong single ion elution profiles are then retained.

RESULTS

Identification of Affinity-purified PHF-Tau Phosphorylation Sites by MS3-data-dependent Neutral Loss Tandem Mass Spectrometry—PHF-Tau immunopurified from human AD brain using the MCI antibody (24) was digested in-solution using trypsin or Lys-C, the trypsin digest was further purified using a phosphopeptide enrichment kit employing immobilized gallium in order to isolate phosphopeptides. Alternatively, PHF-Tau was separated by SDS-PAGE. After visualization, the silver-stained gel was divided into molecular mass regions of 250, 125–250,
Summary of phosphorylated peptides and phosphorylation sites identified in PHF-Tau by LC-MS/MS and MS3

| Peptide | Phosphorylated residues | Score |
|---------|------------------------|-------|
| TPS1PT_PPTT | Ser-217, Ser-214 | 1 |
| TPS1PT_PPTT | Thr-212*, Thr-217* | 1 |
| TPS1PT_PPTT | Ser-217, Ser-214, Ser-194 | 2 |
| TPS1PT_PPTT | Ser-217, Ser-217* | 2 |
| TPS1PT_PPTT | Thr-214 | 2 |
| TPS1PT_PPTT | Thr-212*, Thr-217* | 2 |
| TPS1PT_PPTT | Thr-214 | 2 |
| TPS1PT_PPTT | Thr-212*, Thr-217* | 2 |
| TPS1PT_PPTT | Thr-214 | 2 |
| TPS1PT_PPTT | Thr-212*, Thr-217* | 2 |
| TPS1PT_PPTT | Thr-214 | 2 |

* Residues indicate location on Tau isoform with NCBI accession number NP_005901.
* SEQUEST returned two or more interpretations of the phosphorylation sites on this peptide, as listed.
* (+p) indicates cases number of phosphorylations in cases where phosphorylation sites could not be distinguished from among multiple residues.
* This peptide is only found in 381- and 412-residue Tau isoforms. The sequence of this peptide is equivalent to residues 68-73 of NCBI accession number NP_005901 isoform followed by residues 103-126 of that isoform.

75–125, 60–75, 40–60, 35–40, 30–35, and 25–30 kDa. The gel segment corresponding to each molecular weight region was then subjected to in-gel trypsin digestion as described under “Materials and Methods.” The tryptic fragments resulting from the above procedures were separated by reverse-phase nanoflow capillary high pressure liquid chromatography and analyzed by a linear ion trap tandem mass spectrometer. The combined results from both the in-solution and in-gel digests are summarized below.

The identification of phosphopeptides and phosphorylation sites by tandem mass spectrometry is difficult because the resulting MS/MS spectra are often dominated by an ion corresponding to the precursor ion minus phosphoric acid (which may be termed the neutral loss precursor ion) (28). To improve the quality of phosphopeptide spectra, we took advantage of the NL data-dependent MS3 feature of the LTQ Xcalibur software to trigger an MS3 scan whenever an NL corresponding to loss of H3PO4 (m/z 79.97 for the phosphorylation) was detected. As these spectra illustrate, use of data-dependent NL-triggered MS3 facilitated identification of phosphorylation sites.

Overall, Tau peptides identified by MS/MS covered ~60% of the 66% of the longest Tau isoform (Fig. 2). PHF-Tau peptides with phosphorylation sites identified in this study by LC-MS/MS and MS3 are shown in Table 1. Eight phosphorylation sites (residues) were identified definitively at Ser-214, Thr-217, Thr-231, Ser-235, Ser-237, Ser-262, Ser-396, and Ser-422. Although Ser-214, Thr-217, and Thr-231 are in the proline-rich region of Tau, Ser-235, Ser-237, Ser-262, Ser-396, and Ser-422 lie within the microtubule-binding region.

Several multiply phosphorylated peptides were identified (Table 1, residues 212–221 (b–c); 226–240 (a–d); 386–406 (d); and 407–438 (a–d)). The MS/MS spectra appear in the figure insets, and Table 2 lists the phosphorylation sites identified on these peptides. The MS/MS spectra appear in the figure insets, and Table 2 lists the phosphorylation sites identified on these peptides.

m/z = 529.9 (see inset). The peptide 258SKIGS*pTENLKL67 has an isotopic mass of 1155.54 (the isotopic mass of the peptide (1075.57) plus 79.97 for the phosphorilation). The m/z value of the doubly charged precursor ion is then 578.77. When phosphoric acid is lost from this ion (m/z 49.0 m/z), the m/z value of the resulting product ion is 529.78. The peptide is only found in 381- and 412-residue Tau isoforms. The sequence of this peptide is equivalent to residues 68-73 of NCBI accession number NP_005901 isoform followed by residues 103-126 of that isoform.
FIGURE 3. Identification of PHF-Tau Lys-254 and Lys-311 ubiquitination by LC-MS/MS. Ubiquitination at Lys-254. A, unmodified peptide (243LQTAPVPMPDLK\(^{254}\)); trypsin cleaved at Lys-254. B, ubiquitinated peptide (243LQTAPVPMPDLK*NVK\(^{257}\)); trypsin did not cleave at Lys-254, and a 114-Da two-glycine fragment is conjugated to Lys-254. Ubiquitination at Lys-311. C, unmodified peptide (306VQIVYKPVDLSK\(^{317}\)); trypsin does not cleave at Lys-311 because it is followed by a proline residue. D, ubiquitinated peptide (306VQIVYK*PVDLSK\(^{317}\)); +1 ions extending beyond the modified lysine are shifted by +114 Da in comparison to those of the unmodified peptide.
(a–f)). In most cases the SEQUEST search results indicate two or more possible interpretations of the phosphorylation sites in each spectrum. For example, peptide c of residues 212–221 (212TPSLPTPPTR221) may be phosphorylated either at Thr-212 and Thr-217 or at Ser-214 and Thr-217. In these cases, Table 1 indicates the various modification alternatives. The MS3 spectra of these multiply phosphorylated peptides often have a prominent ion corresponding to the neutral loss precursor ion, as in the MS2 spectra of singly phosphorylated peptides (data not shown). These results indicate potential phosphorylation at Thr-212 and Ser-238 in the proline-rich region and at Tyr-394, Ser-400, Thr-403, Ser-409, Ser-412, Ser-413, Thr-414, and Ser-416 in the microtubule-binding region.

A singly phosphorylated peptide located in the N-terminal acidic region of PHF-Tau was found, although with multiple SEQUEST inter-
pretations of the phosphorylation site, indicating that phosphorylation may occur at Ser-68, Thr-69, Thr-71, Thr-111, or Ser-113. A number of other phosphopeptides were found, as indicated by the presence of MS2/MS3 pairs with neutral losses corresponding to the loss of phosphoric acid. However, the spectra were of insufficient quality to unambiguously verify the identity of the peptide (data not shown).

Identification of Tau Ubiquitination Sites at Lys-254, Lys-311, and Lys-353 by LC-MS/MS

When a protein is ubiquitinated, the C terminus of ubiquitin becomes conjugated to a lysine on the substrate protein by an isopeptide bond. Because the final three residues on the C terminus of ubiquitin are -RGG, when a ubiquitinated protein is digested by trypsin, trypsin will cleave after the arginine residue, leaving behind a

FIGURE 4. Identification of Lys-6, Lys-11, and Lys-48 polyubiquitin linkages by LC-MS/MS analysis. A, polyubiquitination at Lys-48. B, polyubiquitination at Lys-6. C, polyubiquitination at Lys-11.
double-glycine fragment with a molecular mass of 114 Da still attached to the substrate protein. Ubiquitination sites may then be identified by searching for a +114-Da modification at lysine. Ubiquitination also usually results in a missed trypsin cleavage at the modified lysine residue on the ubiquitinated protein (25). We found three ubiquitination sites on PHF-Tau at Lys-254, Lys-311, and Lys-353 (Table 2). All lie within the tubulin-binding domain of Tau; Lys-254 and Lys-353 occur within tubulin-binding segments, whereas Lys-311 lies within a flexible linker segment between tubulin-binding segments (30).

Fig. 3 gives representative spectra for modified and unmodified peptides at ubiquitination sites on PHF-Tau (Lys-254 and Lys-311). The spectra for ubiquitination at Lys-254 illustrate how trypsin fails to cleave at a ubiquitinated lysine residue. Without ubiquitination (Fig. 3A), trypsin cleaves at Lys-254. With ubiquitination, trypsin does not cleave at Lys-254 and the corresponding ubiquitinated peptide is three amino acid residues longer (Fig. 3B). In the case of ubiquitination at Lys-311, the unmodified peptide (Fig. 3C) is not cleaved by trypsin at Lys-311, because Lys-311 is followed by a proline residue (Pro-312). As a result, the unmodified peptide covers the same amino acid span as the ubiquitinated peptide (Fig. 3D). In comparison to the spectrum for the unmodified peptide, the singly charged ions in the spectrum for the ubiquitinated peptide clearly illustrate the shift of +114 Da representing conjugation of the double glycine tail of ubiquitin (see for example, the b-8, b-10, y-9, and y-10 ions).

Identification of Polyubiquitin Linkages at Lys-6, Lys-11, and Lys-48—We identified 72 of 76 amino acid residues in ubiquitin associated with PHF-Tau, and we found three polyubiquitination sites (Lys-48, Lys-11, and Lys-6), suggesting PHF-Tau can be modified by three different forms of polyubiquitination. Fig. 4 shows MS2 spectra for the polyubiquitin linkages at Lys-48 (Fig. 4A), Lys-6 (Fig. 4B), and Lys-11 (Fig. 4C) of ubiquitin. The spectra for polyubiquitination at Lys-11 is accompanied by a diagram of the amino acid sequence of the peptide and the corresponding b and y ions that were identified, which are composed of a mixture of +1 and +2 ions. The peptides found indicating polyubiquitin linkages are listed in Table 3. No ubiquitination sites were found by LC-MS/MS in any of the other proteins (such as actin; see Table 6) that co-precipitated with PHF-Tau in MC1 immunopurification, indicating that the degree of any ubiquitination on these proteins falls below the detection limits of our current methods.

Validation of Polyubiquitination in Affinity-purified PHF-Tau by Immunoblotting Analysis—To biochemically confirm our mass spectrometric data providing evidence of the ubiquitination of Tau, the affinity-purified PHF-Tau sample was subjected to SDS-PAGE analysis followed by Western blot detection with a monoclonal antibody against polyubiquitin. Ubiquitin immunoreactivity was detected in a broad molecular mass range of ~25–250 kDa (Fig. 5, left panel). This is consistent with our proteomic data (Table 6) indicating the statistical validation of the presence of a ubiquitin homolog (ubiquitin B) in-gel slices encompassing proteins of 20–250 kDa (see Fig. 7, which shows the gel image and chromatograms from each gel region). Although there is one nonspecific protein band of about 47 kDa that is detected by both the anti-ubiquitin antibody and the 2° antibody control (Fig. 5, left and right panels, respectively), there are a considerable number of specific ubiquitin-immunoreactive protein bands.

Relative Quantification of Polyubiquitination Levels by SRM—To determine the relative levels of polyubiquitination at Lys-6, Lys-11, and Lys-48, a mass spectrometric instrument method employing SRM was utilized to perform an MS2 scan event for each of the m/z values of the precursor ions of the peptides corresponding to the polyubiquitination sites as well as the m/z values of the corresponding unmodified peptides (see "Materials and Methods" and Table 4 for details). Fig. 6, A–C, shows the SRM base peak ion chromatograms of the six peptides corresponding to polyubiquitination at Lys-6, Lys-11, and Lys-48 for a representative gel segment (60–75-
Polyubiquitination of PHF-Tau

kDa region). Table 5 indicates the relative abundance of these peptides as shown by their peak areas for the same gel segment. Fig. 6D shows the chromatograms for the modified peptides on the same scale for visual comparison of their relative areas. These results suggest that the relative levels of polyubiquitination at the three sites are Lys-48 >> Lys-6 ~ Lys-11. Similar results were obtained for gel segments with mass ranges of 125–250, 75–125, 40–60, 35–40, and 30–35 kDa. Although the absolute ionization efficiencies of the peptides have not yet been determined, these results provide an initial relative quantitative comparison of Tau polyubiquitination levels.

The elution profiles of the unmodified and polyubiquitinated peptides are consistent with the predicted hydrophobicity of the peptides as seen, for example, in the retention times for polyubiquitination at Lys-6 (Fig. 6A); the unmodified peptide (1MQIFVK) has a retention time of 78.5 min, and the polyubiquitinated peptide (1MQIFVK*TLTGK11), which is nearly twice as long, has a retention time of 96.8 min. It should be noted that the unmodified peptide for polyubiquitination at Lys-48 (1LIFAGKQLEDGR52) represents a missed trypsin cleavage and thus is probably only a fraction of the total unmodified ubiquitin at that residue (the two shorter peptides (LIFAGK and QLEDGR) were not observed).

Proteomic Characterization of Proteins Co-precipitating with PHF-Tau—To determine the identities of proteins that co-precipitate with Tau in MC1 immunopurification, an affinity-purified PHF-Tau sample from human AD brain was separated by SDS-PAGE, and the proteins were subsequently visualized by silver staining. The gel was then divided into eight separate regions that were processed by in-gel digestion with trypsin and analyzed via LC-MS/MS. The MS/MS spectra were searched against a human database, and the statistical validation of the resulting peptide and protein assignments was performed through the use of PeptideProphet and ProteinProphet. Portions of the proteins that were identified and statistically validated by this process are listed in Table 6.

Silver staining revealed the presence of several protein bands suggesting the presence of proteins that co-immunopurified with PHF-Tau (Fig. 7). The unmodified isoforms of normal PHF-Tau range in molecular masses from ~37 to 49 kDa (bands in-gel regions 5, 6, and 7), whereas PHF-Tau is known to have reduced electrophoretic mobility and has been reported to appear in three bands at 55, 64, and 69 kDa (31), corresponding to gel regions 4 and 5. The identification of ubiquitin in each of the gel segments augments our MS/MS data establishing the ubiquitination and polyubiquitination of PHF-Tau (Figs. 3 and 4). The identification of tubulin along with Tau implies that Tau largely associates with microtubules under the conditions used to affinity-purify PHF-Tau in this study. The presence of Tau in-gel segment 8 (~20–30 kDa) could indicate the existence of a partially degraded form of Tau, as the lowest molecular mass of the proteins in this gel segment is ~20 kDa, which is less than the molecular mass of the shortest isoform of Tau.

**DISCUSSION**

In this study, we performed a series of functional proteomic and mass spectrometric analyses to characterize the extent of phosphorylation and ubiquitination in heat-stable, soluble PHF-Tau isolated from AD brain by the MC1 antibody, which recognizes a conformational variant of Tau unique to human AD brain (24) that can be surmised to represent an early stage in the generation of PHFs in AD (32, 33). The results show that this conformational variant of Tau has already been subjected to extensive phosphorylation. Phosphorylation at a tyrosine residue (Tyr-394) within the microtubule-binding domain is also indicated by our results, implying that the conformation of Tau isolated for this study could be pathologically important as reported in studies of Tau tyrosine phosphorylation (34). However, peptides corresponding to this tyrosine phosphorylation were low in abundance, and the functional consequences of such modification remain largely elusive. We also found that this early form of PHF-Tau is significantly ubiquitinated. Although it

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**TABLE 3** Polyubiquitination linkages identified in LC-MS/MS analysis of PHF-Tau

| Peptide Linkage | Residues Peptide | Linkage |
|-----------------|-----------------|---------|
| Ub-Lys-6*modified | MQIFVK*TLTGK | Lys-6 |
| Ub-Lys-6*modified | TITLEVEPSDTIENVK | Lys-11 |
| Ub-Lys-48*modified | LIFAGKQLEDGR | Lys-48 |

**TABLE 4** SRM method used in relative quantification of polyubiquitination levels

| Peptide sequence | Precursor ion | MS/MS scan range | Product ion used to construct SRM chromatogram |
|------------------|--------------|-----------------|-----------------------------------------------|
| Ub-Lys-6*modified | 1MQIFVK*TLTGK11 | 765.55 | 400–700 | 506.3 |
| Ub-Lys-11*modified | 2LIFAGKQLEDGR54 | 690.53 | 950–1250 | 1007.7 |
| Ub-Lys-11*modified | TITLEVEPSDTIENVK27 | 894.87 | 950–1250 | 1002.5 |
| Ub-Lys-11*modified | 3LIFAGKQLEDGR54 | 801.66 | 600–1500 | 1115.7 |
| Ub-Lys-48*modified | 4LIFAGKQLEDGR54 | 674.04 | 840–1140 | 618.2 |
| Ub-Lys-48*modified | 5LIFAGKQLEDGR54 | 731.48 | 450–750 | 618.2 |
has been suggested previously that Tau ubiquitination takes place late in AD (35), our results indicate that it may take place at a rather earlier stage. Proteomic analysis also suggested that soluble affinity-purified Tau is still largely associated with tubulin, suggesting there may be a relationship between the post-translational modification of Tau and the alteration of the neuronal function in AD. In addition, a method for mass spectrometric based quantification to examine the extent of ubiquitination was developed. Future use of such assays could lead to further clarification of the roles of Tau modification in modulating neuronal survival and function during the progression of AD.

**PHF-Tau Hyperphosphorylation and Pathological Consequences**

There is a large body of research suggesting that phosphorylation plays a role in regulating the biological functions of Tau. Phosphorylation at Ser-214, Thr-231, and Ser-262 reduces the binding of Tau to microtubules (36–40). Tau phosphorylation is higher in the fetal brain, which has only the shortest Tau isoform, and decreases with age (7). Tau is

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**FIGURE 6.** Relative quantification of Lys-6, Lys-11, and Lys-48 polyubiquitin linkages by SRM. Polyubiquitin linkages at Lys-6 (A), Lys-11 (B), and Lys-48 (C) of ubiquitin (ubiquitinated and unmodified peptides) were quantified by specific precursor-to-product ion transition monitoring (Table 4). D shows chromatograms for all three ubiquitinated peptides on the same scale for visual comparison of the relative peak areas.
differentially distributed in growing axons depending on its phosphorylation state at Ser-202 (41, 42). There is less evidence that Tau phosphorylation is directly related to toxicity. Although Fath et al. (43) showed that pseudo-hyperphosphorylated Tau was cytotoxic in a cell model, suppression of Tau expression alone in a mouse model halted neuronal death (2). Thus it is not clear whether the role of Tau in toxicity and disease pathology is specifically related to its phosphorylation state or to the presence of abnormal Tau aggregates (19, 48). Although in vitro studies have shown that unphosphorylated Tau can form filamentous structures, these studies involved nonphysiological conditions (19, 20); also, Alonso et al. (44) have reported that hyperphosphorylated Tau from AD brain self-aggregates into PHF-like structures.

Previous studies using mass spectrometry have identified phosphorylation sites on PHF-Tau from AD brain isolated by various biochemical methods (45, 46). This study represents for the first time, however, that mass spectrometry has been used to identify PHF-Tau phosphorylation sites using a sample prepared by immunoprecipitation using a monoclonal antibody that recognizes a conformation-dependent epitope of PHF-Tau specific to AD brain. This adds an increased level of confidence that the phosphorylation sites identified here are indeed on forms of PHF-Tau in a conformation unique to AD brain, thereby raising the pathological significance of the results. Unlike other PHF preparations, the PHF-Tau isolated by this method is completely soluble in low concentrations of SDs, although it retains the classical paired helical filament structure by electron microscopy (24).

We succeeded in obtaining peptide coverage of almost all trypsin and Lys-C peptides that could be expected to be seen by the linear ion trap; most of the residues not found would have been represented by peptides either too short or too long for the mass range of the instrument (Fig. 2). The results showed that soluble, heat-stable PHF-Tau in AD brain is indeed hyperphosphorylated. The definitively identified phosphorylation sites included Ser-214, Thr-231, and Ser-262, which, as discussed above, have been implicated in reducing Tau affinity for microtubules. Phosphorylation at Ser-235 was also identified, which is involved in the overall results reported in previous mass spectrometry-based studies (45, 46). This implies that hyperphosphorylation of Tau is an early event that occurs before or during the formation of fibrils, although they are still soluble.

This raises the question whether a conformational change may be a defining event that leads to the formation of fibrils. Previous studies suggest that a bend in PHF-Tau, possibly in the vicinity of Thr-231, results in a Tau conformation in which the N terminus associates with the third microtubule-binding region (49). This is supported by studies by Lim and Lu (50), which show that many features of AD pathology can be related to the levels and functions of Pin1, a peptidyl-prolyl isomerase that specifically performs cis-trans isomerization at Pro-232 of Tau phosphorylated at Thr-231/Ser-235. Furthermore, extensive studies using monoclonal antibodies have linked AD-specific conformational variants of Tau to early pathology in AD (32, 51).

It may also be noted that the use of a data-dependent algorithm to trigger MS3 scans upon detecting a neutral loss of H$_3$PO$_4$ was of clear value in improving the quality of the spectra used in identifying phosphopeptides and phosphorylation sites; in the case of singly phosphorylated peptides, the phosphorylation site could usually then be identified unambiguously. It was often not possible to identify definitively all phosphorylation sites on multiply phosphorylated peptides. Such spectra may represent a hybrid selection of ions of the same peptide variably phosphorylated at different residues, but it is also possible that increasing the MS$n$ level of the analysis to MS4 would enable more sites to be established definitively.

**Polyubiquitination of PHF-Tau**

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### TABLE 5

**Peak areas of modified and unmodified peptides from PHF-Tau polyubiquitination sites**

Using the instrument method of Table 4, peaks obtained by reverse-phase LC-MS/MS analysis of MC1-immunopurified PHF-Tau, with which a substantial amount of ubiquitin co-precipitates, were integrated by ICIS and 15-point Boxcar smoothing using Xcalibur software. Areas are from gel segment with a mass range of 60–75 kDa.

| Ubiquitin, Lys-6 | Ubiquitin, Lys-11 | Ubiquitin, Lys-48 |
|-----------------|------------------|-----------------|
| Area$_{mod}$  a  | 5.9 × 10$^6$      | 3.3 × 10$^6$    | 2.3 × 10$^6$    |
| Area$_{unmod}$ b | 2.0 × 10$^6$      | 8.0 × 10$^3$    | 3.0 × 10$^3$    |

a Area$_{mod}$ indicates peak area of ubiquitinated peptide.
b Area$_{unmod}$ indicates peak area of unmodified peptide.

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### TABLE 6

**Identification of PHF-Tau co-immunoprecipitating proteins**

Shown is a partial list of proteins that co-immunoprecipitate MC1 with PHF-Tau as determined by in-gel digest, LC-MS/MS, SEQUEST search against a human database, and statistical validation by PeptideProphet and ProteinProphet. Probability refers to ProteinProphet probability of correct protein assignment. Ubiquitin B, tubulin α1, and β-actin (boldface) represent groups of homologous proteins among which individual definitive protein assignments could not be made statistically.

| Protein name | NCBI accession number | Probability | Percent coverage | Gel segment number(s) (see Fig. 7) |
|--------------|-----------------------|-------------|-----------------|-----------------------------------|
| Microtubule-associated protein Tau | NP_034968.1 | 1.00 | 36.6 | 1–8 |
| Tubulin, β | NP_076205.1 | 1.00 | 15.1 | 5 |
| Glial fibrillary acidic protein | NP_034477.1 | 1.00 | 14.1 | 6, 7 |
| Creatine kinase | NP_067248.1 | 1.00 | 16.0 | 6 |
| Ubiquitin B | Protein group | 1.00 | 74.8 | 1–8 |
| Tubulin, α1 | Protein group | 1.00 | 14.4 | 1–8 |
| β-Actin, cytoplasmic | Protein group | 0.99 | 5.0 | 5, 6 |
became degradation-resistant or the degradation pathway failed, Tau would accumulate.

Thus it is important to understand the normal in vivo degradation pathway(s) of Tau, and whether accumulation of Tau is because of a failure in Tau degradation. That this is indeed a crucial question is indicated by the results of studies by Keck et al. (23), who demonstrated a decrease in proteasome function in AD and showed that PHF-Tau from AD brain inhibits proteasome activity in vitro.

We found that soluble PHF-Tau isolated by the MC1 antibody from AD brain is ubiquitinated at Lys-254, Lys-311, and Lys-353, all of which lie within the microtubule-binding region. Our results suggest that this ubiquitination occurs as a combination of both mono-ubiquitination and polyubiquitination and show that polyubiquitination occurs mostly at Lys-48 but also at Lys-11 and Lys-6. Moreover, ubiquitination and polyubiquitination occurred throughout the molecular weight range of Tau species present, showing that these modifications may be a marker of degradation resistance.

Because polyubiquitination of proteins by Lys-48-linked ubiquitin chains normally targets proteins for degradation by the UPS, and because it has been shown that Tau is a substrate of the UPS (54), why these polyubiquitinated Tau molecules are not degraded is an important question. The results of this study also raise a number of other questions, such as whether the polyubiquitin chains in question occur as pure Lys-48, Lys-11, and Lys-6 chains, as chains of mixed Lys-48-, Lys-11-, and Lys-6-linked ubiquitin molecules, or as forked chains with ubiquitin linked to more than one residue of the same ubiquitin molecule, and what isoforms of Tau are ubiquitinated.

Chains of the variant ubiquitin UBB\(^{+1}\), which is found in AD and results from a transcriptional error in expression of ubiquitin, have been shown to inhibit the 26 S proteasome and to induce expression of heat-shock proteins. UBB\(^{+1}\) has an elongated C terminus that prevents it from being conjugated to proteins but does not prevent it from being assembled into multi-UBB\(^{+1}\) chains, which bind to the 26 S proteasome (55). Searching our results against a database containing the UBB\(^{+1}\) sequence failed to show any peptides belonging to the elongated C terminus, however. It has been shown that ubiquitin in NFTs is cross-linked to Hsp27, Parkin, and α-synuclein by γ-glutamylation (56). However, we did not find any Hsp27, Parkin, or α-synuclein peptides, indicating that this particular type of cross-linking is a later event. Our data do show a quantifiable amount of Lys-6-linked polyubiquitination. This could be an important early event in the formation of a species of Tau that inhibits the proteasome, because it has been shown that Lys-6-modified ubiquitin (such as by biotinylation) is an inhibitor of ubiquitin-dependent proteolysis (57). It is possible that ubiquitination at Lys-6 could likewise inhibit ubiquitin-dependent proteolysis, representing an event that prevents polyubiquitinated Tau, which would normally be disposed of by the UPS, from being degraded.

This study also demonstrates that qualitative and quantitative determination of PTMs across the Tau molecule can be efficiently achieved by tandem mass spectrometry in the absence of site-specific antibodies. Ubiquitination sites are readily identified, and the use of data-dependent neutral loss MS3 scanning facilitates the identification of specific phosphorylation sites. Preliminary quantitation data indicate that with the use of standards it should be possible to determine definitively the stoichiometry of post-translational modifications as well. Thus a mass spectrometric approach should make it possible to elucidate the temporal sequence of Tau PTMs in samples representing different pathological states of AD and to provide a basis for investigating whether these are related to proteasome inhibition. This in turn may help identify what species of Tau contribute to neuropathology and may even provide a basis for development of high-throughput diagnostic methods for early AD.

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