Unique Alzheimer's Disease Paired Helical Filament Specific Epitopes Involve Double Phosphorylation at Specific Sites†

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ABSTRACT: Alzheimer's disease (AD) paired helical filaments (PHFs), building blocks of neurofibrillary tangles (NFTs) are composed of hyperphosphorylated forms of the microtubule-associated protein τ (i.e., PHF-τ). Currently, much effort is devoted to the development of diagnostic antibodies specific for PHF-τ since elevated τ levels are found in the cerebral spinal fluid of AD patients. To this end, we have mapped the epitopes of a large panel of monoclonal antibodies (mAbs) that recognized only phosphorylation dependent epitopes on PHF-τ. These mAbs include the PHF-τ specific mAb AT10 and 12 newly developed anti-PHF mAbs that recognize PHF-τ but not autopsy-derived normal adult τ on Western-blot and enzyme-linked immunosorbent assay (ELISA). Epitope analysis, together with data on known binding sites of previously published mAbs, revealed that Ser214, Thr231, and Ser396 are immunodominant phosphorylated amino acids in PHF-τ. Six of the 12 new mAbs recognized one of these three phosphorylated sites. With the exception of AT10 and PHF-27, all the mAbs also labeled fetal τ and biopsy-derived τ. Since mAbs AT10 and PHF-27 had little or no affinity for fetal τ and biopsy τ, they can be considered as the first "true" PHF-specific antibodies capable of distinguishing τ isoforms from normal versus AD subjects, suggesting a possible utility of these mAbs as diagnostic markers. Remarkably, the true PHF-specific antibodies recognized peptide sequences phosphorylated on more than one amino acid residue. The peptide recognition of mAb AT10 required the simultaneous phosphorylation of Thr212 and Ser214, and the peptide recognition of mAb PHF-27 was markedly increased when both the primary site Thr231 and the subsite Ser235 were phosphorylated. Since AT10 and PHF-27 are the only mAbs currently available that bind specifically to PHF-τ, these data suggest that double phosphorylation at Thr212/Ser214 and Thr231/Ser235 may be unique to PHF-τ. These data may facilitate the development of mAbs that can be used as specific diagnostic reagents for the detection of altered τ in cerebrospinal fluid of AD patients.

The major histopathological abnormalities that characterize the brains of patients with Alzheimer's disease (AD)[1] as well as those of older individuals with Down syndrome include the excessive presence of neurofibrillary tangles (NFT), and senile plaques (SP) (Wisniewski & Wegiel, 1995; Trojanowski et al., 1995; Yankner, 1996). NFTs are composed of paired helical filaments (PHFs) that are biochemically and structurally distinct from the amyloid fibrils in SP. PHFs and the straight filaments that dominate ultrastructural images of neurofibrillary lesions are likely formed from hyperphosphorylated forms of the low molecular weight microtubule-associated protein, known as PHF-τ (Lee et al., 1991; Goedert, 1993; Trojanowski et al., 1994). In contrast, the major subunit proteins of amyloid fibrils are amyloid β peptides (Aβ), which are about 39−42 amino acids long and are derived from one or more larger, alternatively spliced amyloid precursor proteins (APP) encoded by a gene on chromosome 21 (St George-Hyslop et al., 1987). SP are sites at which abundant accumulations of both PHF and amyloid fibrils converge (Trojanowski et al., 1994). Despite the presence of APP, presenilin 1 and presenilin 2 mutations in rare cases of familial AD (Sherrington et al., 1995; Levy-Lahad et al., 1995), the abundance of NFTs in the brain correlates better with the severity of dementia in AD than the density of amyloid plaques (Dickson et al., 1991; Arriagada et al., 1992; Hyman, 1996).

A major prerequisite for evaluating therapeutic and/or prevention strategies of AD would be the availability of valid biological markers of AD. However, currently there is no diagnostic biochemical test that can be used reliably to stage the cognitive status of AD patients. In this regard, it is significant that the levels of τ protein are increased in the cerebrospinal fluid (CSF) of AD patients, and that the CSF τ levels may correlate with clinical measures of dementia severity (Arai et al., 1995; Mock et al., 1995). Both studies used the very sensitive monoclonal antibody (mAb) AT120 for the detection of τ protein in a sandwiched ELISA system. Unfortunately, mAb AT120 detects normal adult τ and PHF-τ equally well. The development of more specific antibodies that are of high affinity and can distinguish between normal and PHF-τ may further enhance the discriminative properties

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[1] Abbreviations: Aβ, amyloid β peptides; AD, Alzheimer's disease; APP, β-amyloid precursor protein; cAMP-PK, cyclic AMP-dependent protein kinase; CD, circular dichroism; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; MAP, mitogen-activated protein; NFT, neurofibrillary tangles; PHF, paired helical filaments; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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of such assays and this is a major focus of current AD research. A promising mAb, AT10 (Mercken et al., 1992), labels only PHF-τ, and it does not label normal adult-autopsy derived τ, fetal τ, or biopsy-derived τ (Matsuo et al., 1994). Until now, the identification of the epitope recognized by AT10 has been unsuccessful due in part to the use of singly phosphorylated test peptides and inappropriately mutated protein antigens.

The sites of abnormal phosphorylation of PHF-τ are among the most studied and debated aspects of AD. Functionally, τ binds to tubulin and PHF-τ does not (Scott et al., 1992; Bramblett et al., 1993; Yoshida & Ihara, 1993; Biernat et al., 1993). The lack of the microtubule binding of PHF-τ is usually attributed to hyperphosphorylation as tubulin binding can be restored by dephosphorylating PHF-τ (Bramblett et al., 1993; Yoshida & Ihara, 1993). However, τ can form PHFs under *in vitro* conditions without being phosphorylated (Wille et al., 1992; Goedert et al., 1996). The absence of abnormal hyperphosphorylation of τ in intracellular tangles of AD was also noted by studying the staining of NFTs with supposedly phosphate-specific mAbs, such as AT8, AT180, AT270, SMI31, and BR133 (Bondareff et al., 1995). In reality, however, these antibodies may not ultimately be specific for PHF-τ (Matsuo et al., 1994; Goedert et al., 1994). The conclusive test whether an antibody is truly specific for PHF-τ is the inability of the mAb to bind fetal τ and biopsy-derived τ. Fetal τ and biopsy-derived τ were shown to contain more phosphate groups than autopsy-derived τ (Matsuo et al., 1994; Garver et al., 1994, 1996; Singh et al., 1996). Dephosphorylation of PHF-τ can be very fast at some sites (Seubert et al., 1995), giving rise to the possibility of unrealistic immunoreactions with antibodies directed against these sites. Biopsy-derived τ refers to preparations of τ isolated from biopsy brain samples of healthy individuals seconds after acquisition, and τ is not exposed to the extremely fast dephosphorylation by endogenous phosphatases in the tissues. Our aim here was to develop diagnostic antibodies that distinguish PHF-τ from biopsy-derived τ, fetal τ, and autopsy-derived τ. In this regard it is not important whether the extra phosphates are the ingenious cause of the development of PHF or are added to the protein during or after the deposition process.

An increasing amount of evidence suggests that mAbs obtained after immunization with PHF-τ recognize multiphosphorylated protein fragments. As the previous investigations used singly phosphorylated synthetic peptides or mutated protein constructs to identify the binding sites of these mAbs, this may explain why the minimal or close-to-minimal epitopes and exact phosphate requirements of many of the antibodies have not been characterized. In the current study we used highly purified synthetic peptides as well as singly and doubly phosphorylated analogues to identify the binding sites of AT10 (Mercken et al., 1992) and of 12 newly developed mAbs to PHF-τ proteins. The epitope analysis of the new mAbs that detect PHF-τ revealed immunodominant phosphoserine and phosphothreonine residues in PHFs. One of the newly developed mAbs, PHF-27, just like mAb AT10, labels only PHF-τ.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized on Milligen 9050 and Rainin PS3 automatic synthesizers using 9-fluorenylmethoxycarbonyl amino acids according to standard procedures (Fields & Noble, 1990). Phosphoserine or phosphothreonine residues were incorporated as Fmoc-Ser/Thr(PO_3HBzl)-OH (Wakamiya et al., 1994), purchased from Novabiochem, Ltd. Peptides and phosphopeptides were detached from the solid support with trifluoroacetic acid (TFA), and they were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using an aqueous acetonitrile gradient elution system containing 0.1% TFA as an ion pairing reagent. The integrity of the peptides and phosphopeptides was verified by mass spectroscopy. Table 1 lists the synthetic peptides.

Enzyme-Linked Immunosorbent Assay (ELISA). Direct ELISA was applied to look for regular or conformation-sensitive epitopes (Lang et al., 1994; Otvos & Szendrei, 1996). Briefly, binding of 0.002−5 μg amounts of peptide or phosphopeptide antigens was tested with various dilutions of mAbs on 96-well Nunc Immuno MaxiSorp plates. The antigens were dissolved in water or organic solvents, such as trifluoroethanol (TFE), methanol, or acetontrile, and dried onto the wells at 37 °C overnight. All subsequent steps were performed according to general ELISA protocols (Goding et al., 1986). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was used as secondary antibody at 1/1000 dilution. Color development was made with 3,3′,5,5′-tetramethylbenzidine dihydrochloride and stopped with 2 M aqueous H_2SO_4, and absorption was measured at 450 nm. Purified autopsy-derived normal τ and PHF-τ at 5 μg/ml concentration were used as controls and processed for ELISA as described above. To provide a realistic picture of the performed assays, the background in Figure 1 and Figures 3−7 is not subtracted.

Immunization with PHFs. Highly purified PHF-τ preparations were prepared as described (Lee et al., 1991). Immunization with PHF-τ and fusion with mouse myeloma cells SP2/0-Ag14 followed a previously published protocol (Lee et al., 1987). The mAbs were first screened by ELISA and characterized by Western blotting (see next paragraph) and immunohistochemistry using brain sections from AD and control patients.

Western Blot Analysis. Western blotting using autopsy-derived normal adult τ, fetal τ, PHF-τ, and biopsy-derived τ was performed as described earlier (Matsuo et al., 1994). Briefly, nitrocellulose replicas of gels containing electrophoretically separated τ samples were prepared from 10% sodium dodecyl sulfate−polyacrylamide gels and probed with the primary antibodies. MAb binding was detected as previously described (Lee et al., 1991). Protein concentrations in the samples were determined using bicinchonic acid as a dye reagent with bovine serum albumin as the standard (Smith et al., 1985). The amount of protein loaded depended on the different τ preparations which varied from 1.5 to 12 μg. The protein concentrations were adjusted to obtain approximately equally strong immunoreactivity (if binding occurred at all) of the various samples, based on binding of the same preparations to phosphorylation-independent antibody standards T14/46 (Matsuo et al., 1994). Each experiment was repeated at least three times.

Circular Dichroism (CD). CD spectra were taken on a Jasco J720 instrument at room temperature in a 0.2 mm path length cell. Double-distilled water and spectroscopy grade trifluoroethanol were used as solvents. The peptide concentration was about 0.5 mg/mL, determined each time by

Table 1: Synthetic Peptides and Their Characterization[a]

peptides	sequence	retention time (min)	$[M + H]^+$ calculated mass	$[M + H]^+$ observed mass
207-222	GSRSRTPSLPTPPTRE	23.8	1739	1739
212P	T*	23.6	1819	1821
214P	S*	23.6	1819	1819
217P	T*	24.2	1819	1820
212P, 214P	T*S*	23.1	1899	1898
212P, 217P	T* T*	23.9	1899	1900
214P, 217P	S* T*	23.6	1899	1898
224-240	KKVAVVRTPPKSPSSAK	19.7	1780	1780
231P	T*	20.0	1860	1860
235P	S*	19.8	1860	1860
231P, 235P	T* S*	19.9	1940	1940
390-408	AEIVYKSPVVSGDTSPRHL	25.5	2055	2055
396P	S*	24.1	2135	2134
400P	S*	24.9	2135	2134
403P	T*	25.1	2135	2136
404P	S*	25.3	2135	2136
396P, 400P	S* S*	25.4	2215	2214
396P, 403P	S* T*	24.6	2215	2217
396P, 404P	S* S*	24.3	2215	2214
400P, 403P	S* T*	25.3	2215	2215
400P, 404P	S* S*	27.3	2215	2214
403P, 404P	T*S*	32.0	2215	2214

[a] The asterisk(s) denotes phosphorylation sites.

quantitative RP-HPLC (Szendrei et al., 1994). Curve smoothing was accomplished by the algorithm provided by Jasco. Mean residue ellipticity ($[\theta]_{MR}$) is expressed in cm^2 deg/dmol by using a mean residue weight of 110. Because the secondary structures of the peptides (especially phosphopeptides) provided by the current computer-assisted curve analyzing algorithms show a high error rate, the CD spectra evaluations were based on comparison with known peptide conformations (Woody, 1985; Otvos, 1996).

RESULTS

Immunization of mice with PHF-τ yielded a number of mAbs that were tested for their antigen recognition by Western-blot and ELISA. Eleven of the 21 mAbs failed to recognize autopsy-derived normal τ, but did bind to PHF-τ (Figure 1). The antigen specificity of these mAbs was further analyzed by Western-blot. The antigens for Western-blot included normal adult autopsy-derived τ, fetal τ, and PHF-τ. As controls, three existing mAbs were added to the mAb pool: AT10 (Mercken et al., 1992), PHF-1 (Greenberg et al., 1992), and AT180 (Goedert et al., 1994). PHF-1 and AT180 have been reported to label fetal τ and PHF-τ but not autopsy-derived normal adult τ (Matsuo et al., 1994). In contrast, mAb AT10 labels only PHF-τ on Western-blot (Matsuo et al., 1994). Among the newly developed mAbs, only mAb PHF-27 bound specifically to PHF-τ and not to fetal τ, normal adult autopsy τ or biopsy-derived τ (Figure 2). These results indicate that like the mAb AT10, mAb PHF-27 is one of the first "true" PHF-specific antibodies.

The antibodies were tested for their binding to three synthetic peptide families. The peptide groups were selected based on previous published mass spectrometry data on PHF-τ (Watanabe et al., 1993; Morishima-Kawashima et al., 1995) demonstrating regions with clusters of phosphate sites along the length of PHF-τ. All three peptide groups contained unphosphorylated, monophosphorylated and diphosphorylated peptides (Table 1). Of the new 12 mAbs tested, six recognized phosphoamino acids in one of the three peptide families above (Table 2). This observation together with mAbs with previously identified epitopes suggest the

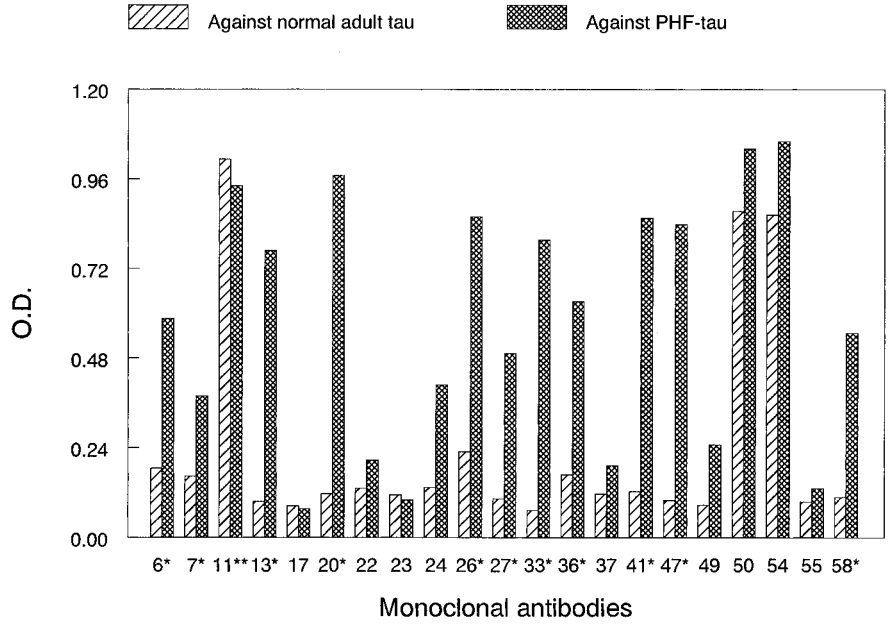

FIGURE 1: Selection of PHF-specific antibodies by ELISA. The diagonally hatched bars indicate the recognition of the newly developed mAbs against normal τ. The cross-hatched bars indicate the recognition of the mAbs against purified PHF-τ. The single asterisks indicate the mAbs we selected for further studies. PHF-11 was also selected because high background was obtained and (on the basis of the first screening) its PHF-specificity could not be established (double asterisk).

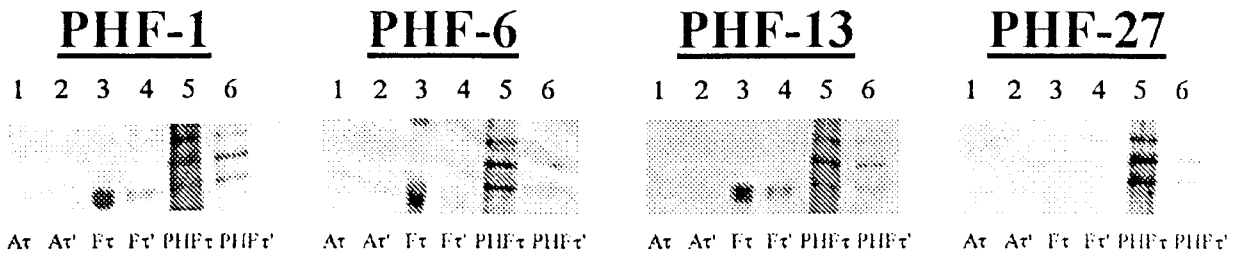

FIGURE 2: Binding of monoclonal antibodies to different τ preparations on Western-blot. The antigens are as follows: lanes 1 and 2 (Aτ): normal adult (autopsy-derived) τ; lanes 3 and 4 (Fτ): fetal τ; lanes 5 and 6: PHF-τ. In lanes 2, 4, and 6 the loaded protein amounts were decreased 10-fold. Biopsy-originated τ preparations were recognized approximately at similar level to fetal τ.

Table 2: Immunodominant Phosphorylation Sites in PHF-τ

mAb	recognized primary phosphate site	secondary phosphate site
	New Antibodies	
PHF-20.6	Ser214	Thr217
PHF-6	Thr231	
PHF-41	Thr231	
PHF-27	Thr231	Ser235
PHF-13	Ser396	
PHF-47	Ser396	
	Existing Antibodies for Which the Exact Epitopes Were Characterized in This Study	
AT10	Thr212 and Ser214 together	
AT180	Thr231	
	Literature Data on the Same Sites[a]:	
M4	Thr231[b]	
PHF-1	Ser396	Ser404[c]
AD-2	Ser396	Ser404[d]

[a] This list contains only mAbs generated by immunizing with PHF-τ protein. [b] Hasegawa et al. (1993). [c] Otvos et al. (1994). [d] Buee-Scherrer et al. (1996).

presence of at least two major immunodominant residues i.e., phosphorylated Thr231 and Ser396 (Table 2).

Previous mass spectrometry studies indicate that both Thr231 and Ser235 are phosphorylated residues of PHF-τ (Morishima-Kawashima et al., 1995). To evaluate whether or not phosphorylated Thr231 alone or Thr231 and Ser235 together are the immunodominant epitope, we synthesized the 224−240 peptide, the two monophosphorylated analogues (231P and 235P), and the diphosphorylated peptide (231P, 235P) in a way which places the phosphoamino acids in the middle of the molecule (Table 1). We wanted to make peptides long enough to cover the entire epitope and possess some secondary structure but short enough to allow preparation with the highest possible purity and not to have more than two conformational elements. MAb AT180 did not bind either to the unphosphorylated peptide 224−240 or the phosphorylated version 235P but did bind to both the same peptide phosphorylated at Thr231 and the diphosphorylated peptide 231P, 235P (Table 2). Once Thr231 was phosphorylated, the antigen recognition of mAb AT180 was not increased after phosphorylation of Ser235. MAb AT180 bound to the Thr231 phosphorylated peptide very strongly. As little as 40 ng of phosphopeptide could be clearly detected at antibody dilution of 1:3600. Thus, mAb AT180 is specific for phosphorylated Thr231 only.

The antigen recognition pattern of two of the new antibodies, mAb PHF-6 and PHF-41, was completely identical to that of mAb AT180. MAb PHF-6 and PHF-41 did not recognize the unphosphorylated or Ser235 phosphorylated 224−240 peptide and recognized the Thr231 phosphorylated and diphosphorylated peptides equally well (Figure 3 and Table 2). In contrast to these antibodies, the antigen

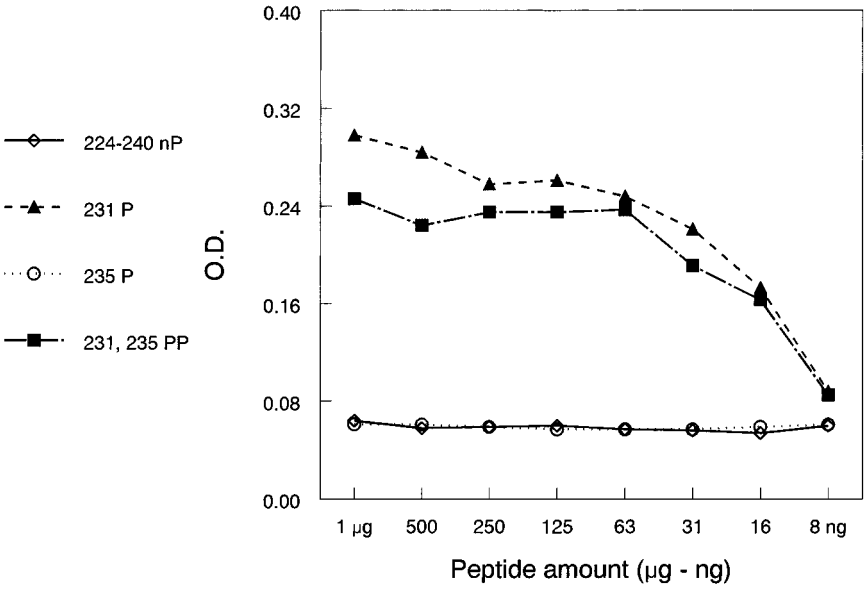

FIGURE 3: Peptide recognition of mAb PHF-6. The ELISA plate was coated with 8 ng to 1 μg of antigens dissolved in water. Solid line (open diamonds), unphosphorylated peptide τ 224−240; dashes (closed triangles), peptide phosphorylated on Thr231; dots (open circles), peptide phosphorylated on Ser235; dots and dashes (closed squares), peptide phosphorylated on both residues. Undiluted supernatant was used.

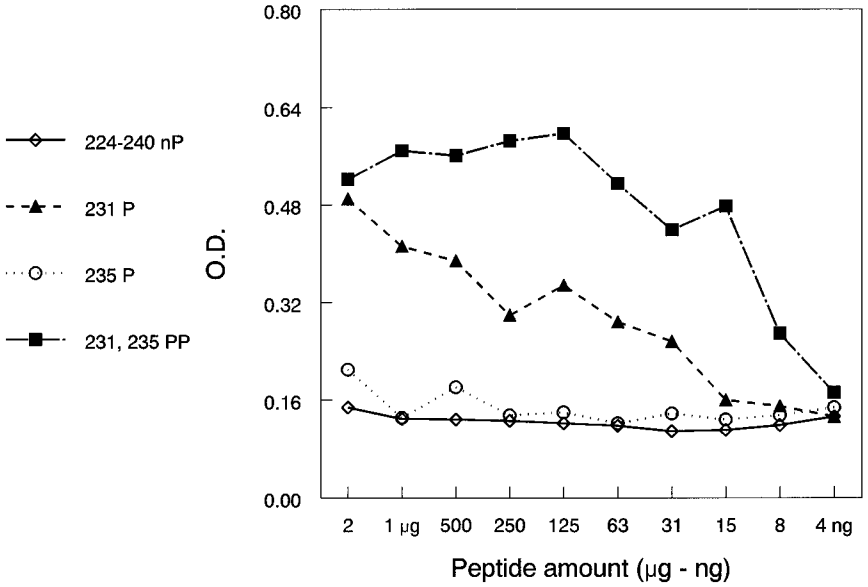

FIGURE 4: Peptide recognition of mAb PHF-27. The ELISA plate was coated with 4 ng to 2 μg of antigens dissolved in water. Solid line (open diamonds), unphosphorylated peptide τ 224−240; dashes (closed triangles), peptide phosphorylated on Thr231; dots (open circles), peptide phosphorylated on Ser235; dots and dashes (closed squares), peptide phosphorylated on both residues. Undiluted supernatant was used.

recognition of mAb PHF-27 showed a different pattern. This mAb did not bind to the unphosphorylated 224−240 peptide, as expected. Like mAb PHF-6, PHF-41, and AT180, mAb PHF-27 recognized singly phosphorylated Thr231 as a main site and did not recognize singly phosphorylated Ser235. However, when both Thr231 and Ser235 were phosphorylated, the antigen recognition was markedly increased, especially when small amounts of antigens were loaded (Figure 4).

MAbs PHF-47 and PHF-13, just like PHF-1, did not recognize unphosphorylated peptide 390−408 or the same peptide phosphorylated at Ser400, Thr403, or Ser404 but did recognize the Ser396 phosphorylated peptide and all diphosphorylated peptides containing phosphorylated Ser396. None of the diphosphorylated peptides lacking phosphate group on Ser396 was notably recognized by these mAbs (Table 2). MAb PHF-20.6 (subclone 6 of antibody 20) did not bind to the unphosphorylated peptide 207−222 or to the peptide phosphorylated at Thr212 (212P). Weak binding at high peptide loads was observed for monophosphorylated peptide 217P and diphosphorylated peptide 212P, 217P. The antibody binding was strong to Ser214 monophosphorylated peptide (214P) (Table 2) but was not significantly increased after incorporation of a second phosphate group to Thr212 or Thr217. Thus, this detailed epitope analysis indicated that mAb PHF-20.6 recognized phosphorylated Ser214 as a main site and the antibody recognition was only slightly modified by the status of phosphorylation of the surrounding subsites.

After identifying the immunodominant phosphoamino acids in PHF-τ , we used the three peptide families to identify the epitope of mAb AT10. Since earlier strategies using monophosphorylated peptides and phosphorylated recombi-

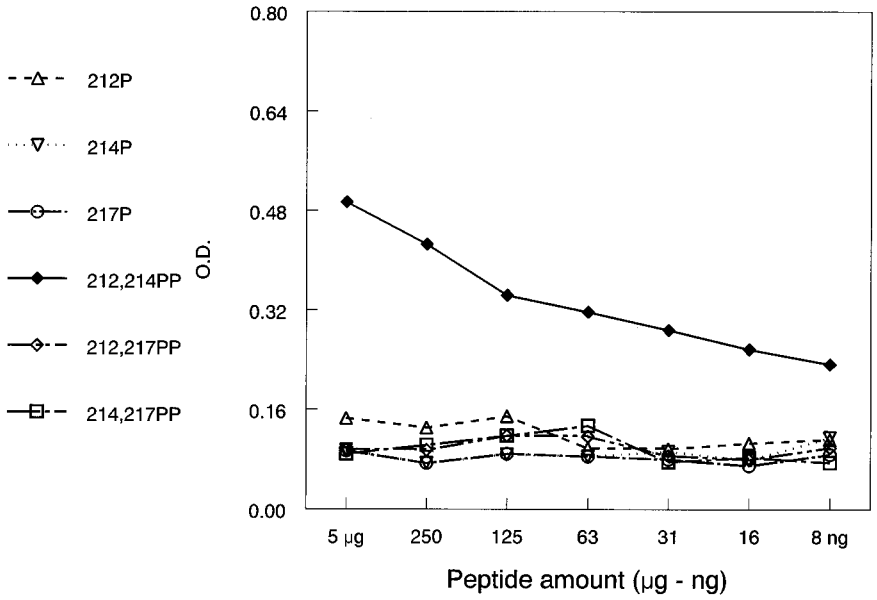

FIGURE 5: Identification of the epitope for mAb AT10. The ELISA plate was coated with 80 ng to 5 μg of monophosphorylated or diphosphorylated τ 207−222 peptides dissolved in water. The mAb was diluted to 1:300. Only the Thr212, Ser214 diphosphorylated peptide was recognized by the mAb (solid line, closed diamonds).

nant τ proteins failed to identify the antigen binding site for this mAb, we concentrated on diphosphorylated peptides. Indeed, no antibody binding was detected to any of the 12 unphosphorylated or monophosphorylated synthetic peptides, but the mAb bound to the antigen when both Thr212 and Ser214 were simultaneously phosphorylated (Figure 5). Interestingly, this mAb failed to recognize the other two diphosphorylated versions of the same peptide, namely those that carried phosphate groups on Thr212 and Thr217 or on Ser214 and Thr217. These data, together with those obtained for mAbs PHF-27, clearly indicate that PHF-specific epitopes include doubly phosphorylated Thr212/Ser214 and Thr231/Ser235.

We asked what factor the "true" PHF-specific mAb PHF-27 recognized and the less PHF-specific mAbs PHF-6, PHF-41, and AT180 did not. All three antibodies bound to phosphorylated Thr231 and did not bind to individually phosphorylated Ser235. To find an answer, we plated the peptides that were recognized by the antibodies [i.e., 231P and 231P, 235P (marked PP in Figures 6 and 7)] for the ELISA from water and also from organic solvents. Various alcohols (Conio et al., 1970) and acetonitrile (Otvos et al., 1993) are often used as cell environment-mimicking solvents to reduce the dielectic constant of purely aqueous solutions. From these organic co-solvents TFE, a structure stabilizer/promoter (Lehrman et al., 1990), is used preferentially for synthetic peptides, mostly because of TFE's excellent solubilizing properties. The peptides and phosphopeptides of this study appeared to be fully soluble in all three organic solvents (i.e., TFE, methanol, and acetonitrile) used. With mAbs PHF-6, PHF-41, and AT180 the antigen recognition was somewhat increased for both peptides when the conformation of the phosphopeptides was stabilized in TFE (Figures 6A and 7). This indicates that these antibodies recognized a phosphopeptide conformation that could be stablized/generated in TFE. The peptide recognition of mAb PHF-6 was not noticeably increased when the antigens were plated from methanol or acetonitrile instead of water, suggesting that TFE was able to present a peptide conformation for mAb PHF-6 that methanol or acetonitrile could not (Figure 6A). When the antigen recognition of mAb PHF-27 was studied, the recognition was dramatically increased for the monophosphorylated peptide plated from TFE or methanol, especially at high antigen loads (Figure 6B). Interestingly, this increase was much less dramatic for the already strong binder diphosphorylated peptide (Figure 6B). This finding suggested that the reason why the antigen recognition by the antibody was already strong toward to the diphosphorylated peptide in aqueous solutions was that mAb PHF-27 saw both the presence of the phosphate group at Thr231 and the conformation of the diphosphorylated peptide that it assumed in water. It is worth noting that similarly to mAb PHF-27, the antigen recognition of the other "true" PHF-specific antibody, AT10 did not significantly increase when the diphosphorylated peptide antigen was plated from TFE, just the "pro-zone" binding was shifted to lower antigen amounts (data not shown).

To explain the differences in antigen recognition, we studied the conformation of the τ 224−240 peptides in water and TFE by circular dichroism (CD). In water all four peptides exhibited type U spectra, characteristic of peptides without conformational preferences (Woody, 1985). The lack of the small positive band around 220 nm indicated the presence of some ordered structures (Figure 8A). On the basis of the CD of the peptides in TFE, this residual ordered structure was likely to be some turn conformation. The peptides exhibited type C CD spectra in TFE (Figure 8B), characteristic of type I β-turns (Smith & Pease, 1980) or mixtures of β-turns with dominant type I character (Perczel et al., 1993). Among the four peptides, the turn character was far stronger for the diphosphorylated peptide than for either of the unphosphorylated or the monophosphorylated variants.

DISCUSSION

It is unclear whether excessive kinase or insufficient phosphatase activities transform normal τ to PHF-τ (Seubert et al., 1995; Matsuo et al., 1994; Goedert et al., 1994), but regardless of the origin of the extra phosphate groups or level

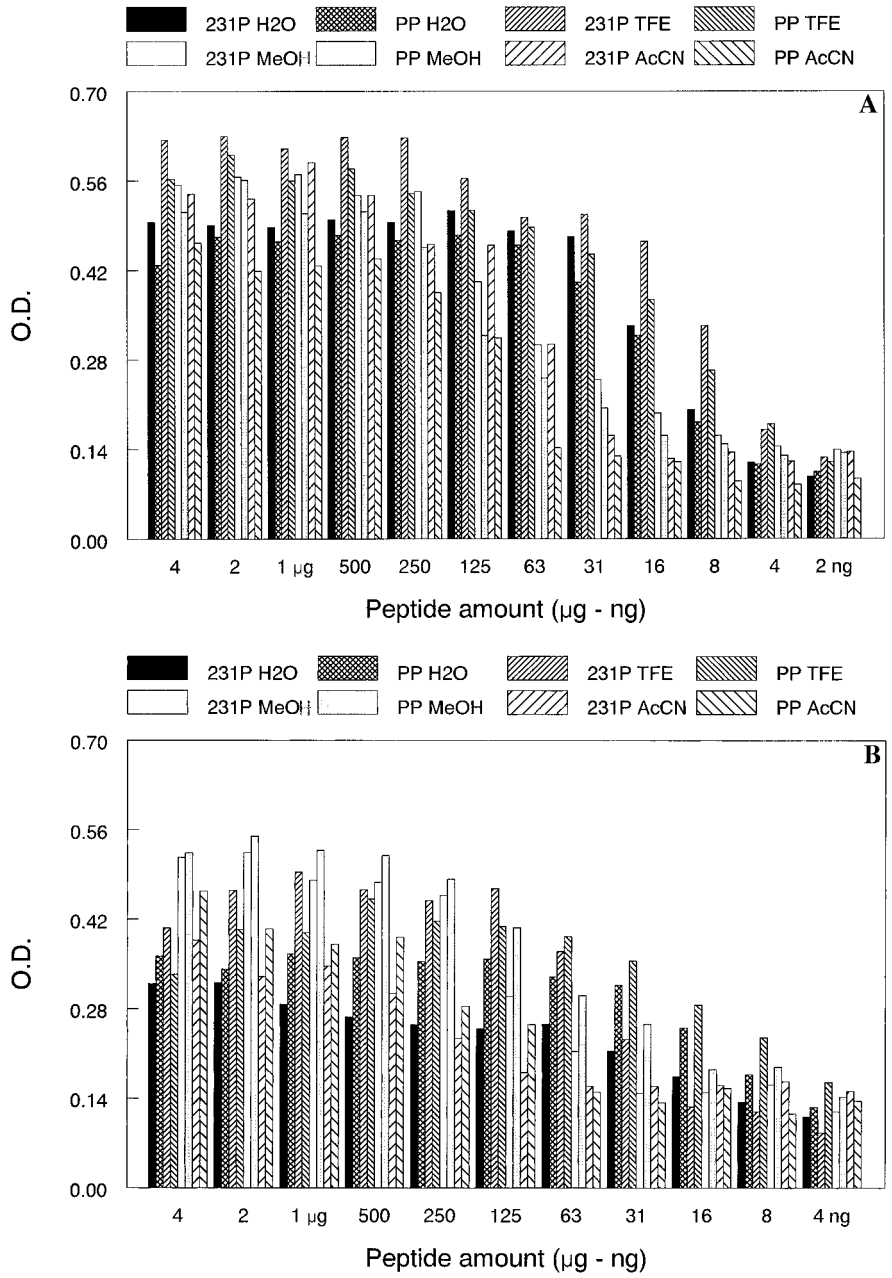

FIGURE 6: Dependence of antibody binding upon the conformation of the antigens. The peptides were applied to the ELISA plate in either water or TFE, methanol, or acetontrile and dried to the plastic. Solid black bars, Thr231 monophosphorylated peptide applied from water; cross-hatched bars, Thr231, Ser235 diphosphorylated peptide applied from water. The closely hatched bars (diagonal stripes) represent phosphopeptides plated from TFE: left, Thr231 monophosphorylated peptide; right, Thr231, Ser235 diphosphorylated peptide. White bar, Thr231 monophosphorylated peptide plated from methanol; gray bar, Thr231, Ser235 diphosphorylated peptide plated from methanol. The sparsely hatched bars represent phosphopeptides plated from acetonitrile: left, Thr231 monophosphorylated peptide; right, Thr231, Ser235 diphosphorylated peptide. Acetonitrile seemed to attack the surface of the plates, but this did not influence the ELISA readings. (A) Primary antibody is PHF-6. (B) Primary antibody is PHF-27.

of phosphorylation on the same sites in PHF-τ, antibodies are used to distinguish between normal τ or PHF-τ (Lee et al., 1991; Lichtenberg-Kraag et al., 1992; Lang et al., 1992; Goedert et al., 1994). Many investigators have been concerned with the extent of PHF-τ phosphorylation and thus with the potentially involved protein kinases and/or phosphatases (Seubert et al., 1995; Morishima-Kawashima et al., 1995; Liu et al., 1994). These studies resulted in controversial assignments of the abnormal sites, mostly due to the use of τ isolated from biopsy brain samples with no postmortem delay (Matsuo et al., 1994; Garver et al., 1994) or from autopsy-derived brain tissue (Hasegawa et al., 1992; Morishima-Kawashima et al., 1995). For example, the phosphorylation of Ser262 (in the microtubule-binding domain) is thought to be a pathologic phosphorylation site specific for PHF-τ (Biernat et al., 1993). However, Ser262 was identified recently as a normal phosphorylation site in τ (Seubert et al., 1995), although it was found previously to be phosphorylated only in PHF-τ (Morishima-Kawashima et al., 1995). This discrepancy is due to the rapid dephosphorylation of Ser262 by brain phosphatases during postmortem delay (Seubert et al., 1995).

Previous studies using mass spectrometry have identified Ser208, Ser210, Thr212, Ser214, Ser262, Thr403, Ser412, and Ser422 as abnormal phosphorylation sites in PHF-τ. However, these mass spectrometry results contradict the results presented here. By using a panel of mAbs directed to PHF-τ, we positively identified the presence of phosphate

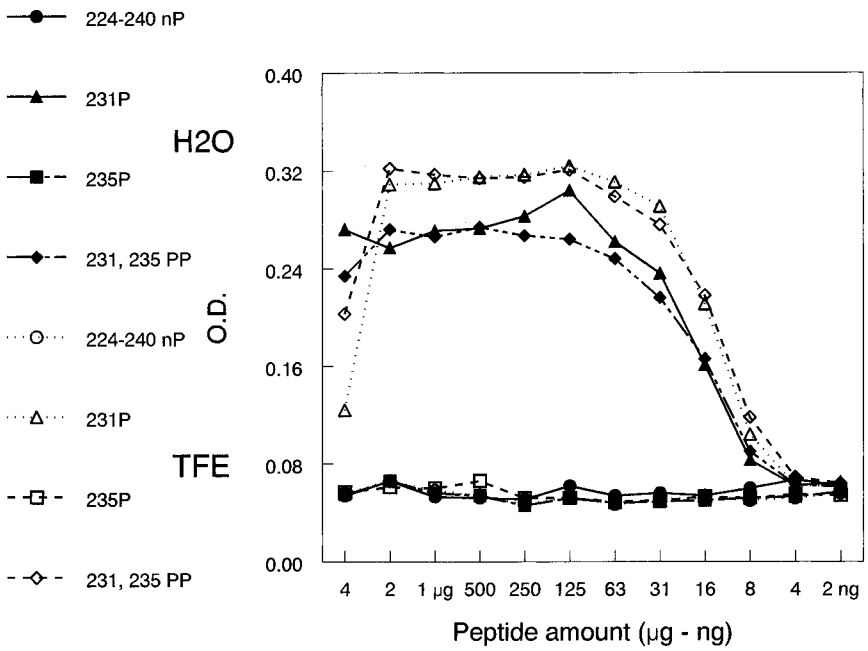

FIGURE 7: Peptide recognition of mAb PHF-41. The ELISA plate was coated with 2 ng to 4 μg of antigens dissolved in water (closed symbols) or TFE (open symbols). Circles, unphosphorylated peptide τ 224−240; triangles, peptide phosphorylated on Thr231; squares, peptide phosphorylated on Ser235; diamonds, peptide phosphorylated on both residues. The dilution of the supernatant was 1:1.

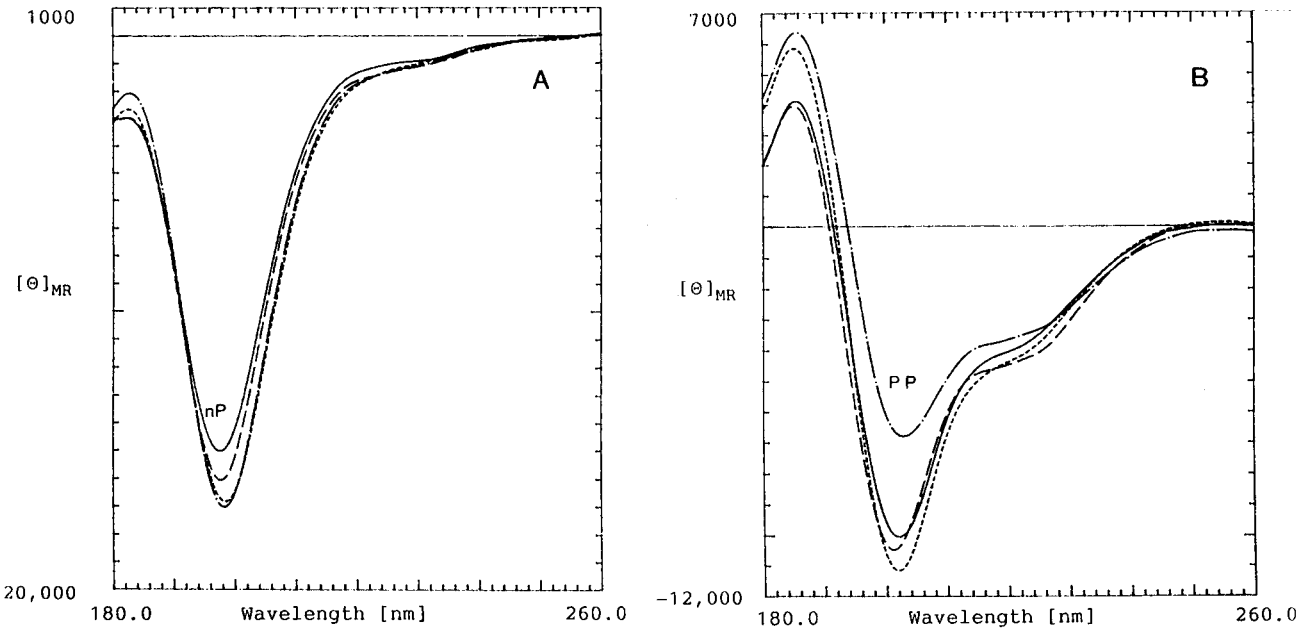

FIGURE 8: CD spectra of τ 224−240 peptides in water (panel A) and in TFE (panel B). Solid line, unphosphorylated peptide; dots, Thr231 phosphorylated peptide; dashes, Ser235 phosphorylated peptide; dots and dashes, diphosphorylated peptide.

group on Ser214 of fetal τ and the concurrent presence of phosphates on Thr212 and Ser214 as well as on Thr231 and Ser235 of PHF-τ. MAb PHF-20 labels fetal τ but not normal adult τ on Western-blot, indicating that Ser214 carries a phosphate in immature brain. On the other hand, both Thr212 and Ser214 needed to be phosphorylated for any recognition by mAb AT10, and both Thr231 and Ser235 needed to be simultaneously phosphorylated for full recognition by mAb PHF-27. MAb PHF-27 did not recognize normal adult τ or fetal τ and very insignificantly biopsy-originated τ. MAb PHF-27 appears to be one of the first true PHF-specific antibodies and a possible reagent for the development of AD-specific diagnostic markers. The binding of PHF-27 to PHF-τ in CSF remains to be determined, albeit the low dilution of this antibody needed for ELISA and Western-blot indicates that PHF-27 may not be the final solution for this purpose. Our preliminary results with the corresponding anti-diphosphorylated peptide antisera indicate that the same specificity, but considerably higher sensitivity, can be achieved by using multiphosphorylated synthetic peptide immunogens.

Goedert and co-workers (1994) demonstrated that *in vitro* Ser235 needs to be phosphorylated before Thr231 can be phosphorylated. They showed that τ can be phosphorylated at Thr231 by glycogen synthase kinase 3 only if Ser235 is already phosphorylated by mitogen-activated protein (MAP) kinase. If this is indeed true, our results suggest that Ser235 must be rapidly dephosphorylated since PHF-27 which binds to both Thr231 and Ser235 does not recognize fetal τ or biopsy-derived τ. However, since PHF-27 binds to PHF-τ,

this supports the notion that specific phosphatases may be hypoactive in the AD brain (or alternatively that kinases may be hyperactive) and are unable to dephosphorylate Ser235 (or rephosphorylate this residue). Recently, Ser235 was shown to be a preferential substrate site for protein phosphatase 2B (Gong et al., 1994) and phosphorylated Thr231 was shown to be an unusually resistant residue to dephosphorylation (Hasegawa et al., 1992). This interpretation is consistent with the current dynamic multiple kinase/phosphatase disequilibrium theory that explains how τ becomes hyperphosphorylated in PHF leading to PHF-τ's inability to bind and stabilize microtubules (Bramblett et al., 1993, Matsuo et al., 1994).

While Thr231 and Ser235 can be phosphorylated by a number of kinases *in vitro* (Morishima-Kawashima et al., 1995), Thr212 and Ser214 are substrates for specific kinases. Thr212 can only be phosphorylated by MAP kinase (Drewes et al., 1992) and brain kinase (Biernat et al., 1993), and Ser214 can only be phosphorylated by cyclic AMP-dependent protein kinase (cAMP-PK) (Scott et al., 1993). Nevertheless, this site is a major target for cAMP-PK: 50% of phosphate incorporated into τ by cAMP-PK is on Ser214 (Litersky et al., 1996) and phosphorylation of Ser214 is one of the major factors responsible for decreasing the ability of τ to nucleate microtubules (Brandt et al., 1994). However, since neither MAP kinase nor cAMP-PK has been shown to phosphorylate τ at these sites in intact cells, the identity of the kinase(s) that phosphorylate PHF-τ to generate the AT10 epitope in the AD brain remains to be identified.

Our results based on the epitope analysis of PHF-specific mAbs AT10 and PHF-27 provide further support to those reported earlier (Seubert et al., 1995). It was suggested that phosphorylation of yet another crucial residue, Ser262 itself is not sufficient to eliminate the binding of τ to microtubules, and simultaneous phosphorylation of multiple sites may be required for the disruption of the interaction between τ and microtubules. Additional proof for multiphosphorylated sequences in PHF-τ is the finding that prephosphorylation of τ by non-proline-dependent kinases dramatically increases the rate of further phosphorylation by proline-dependent kinases (Singh et al., 1995). Since substrate serines and threonines of the two types of enzymes are often located one or two amino acid residues away from each other, it is reasonable to suppose that multiphosphorylation sites other than the Thr212/Ser214 or Thr231/Ser235 sites are present in PHF-τ. We prepared multiphosphorylated peptides corresponding only to the regions expected to be immunodominant, or abnormally phosphorylated in PHF-τ. By using these peptides we identified the binding sites of six of the 12 PHF-specific antibodies. It is reasonable to suppose that the rest of the antibodies, for which the epitopes are currently unknown, recognize multiphosphorylation domains different from those we studied. Alternatively, these mAbs may recognize post-translational modifications other than phosphorylation. Although earlier mass spectroscopy studies had failed to identify covalently linked carbohydrates in normal τ or PHF-τ (Watanabe et al., 1993; Morishima-Kawashima et al., 1995), recently PHF-τ was reported to carry complex N-linked carbohydrates while normal adult τ was not (Wang et al., 1996). In another paper, O-linked N-acetylglucosamine attachment to bovine τ was demonstrated by blotting with succinylated wheat germ agglutinin and by probing with bovine milk galactosyl transferase (Arnold et al., 1996).

Full-size normal human τ possesses only a negligible fraction of periodic (α-helix or β-pleated sheet) conformations in solution (Cleveland et al., 1977), and this is independent of whether τ is obtained from the brain or expressed in bacteria, and whether it is in its endogenous state of phosphorylation or free of phosphate (Wille et al., 1992). Schweers and co-workers have studied the conformation of normal human τ and PHF-τ by CD, Fourier-transform infrared spectroscopy, scattering and imaging methods (Schweers et al., 1994). No detectable ordered secondary structure was found. The authors conclude that it is unlikely that the aggregation of τ into AD PHF is based on interactions between strands of β-sheets. Instead, the cross-β pattern of the earlier tangle preparations could have arisen from the Aβ component. Accordingly, none of the 22 synthetic peptides we studied here exhibited any β-sheet component by CD. Instead, these peptides exhibited CD spectra indicative of various equilibriums of unordered and turn conformers. Full-sized τ is an elastic molecule (Cleveland et al., 1977), and indeed, CD shows that the major epitopes of both normal τ and PHF-τ assume unordered structure in water and β-turn conformation in TFE solutions (Szendrei et al., 1993; Lang et al., 1992). This in turn, may indicate that mAb PHF-27, as opposed to mAbs PHF-6, PHF-41 and AT180, saw not only the presence of the phosphate group, but also the residual turn conformation of the diphosphorylated peptide in water. CD studies of the differentially phosphorylated peptides supported this hypothesis.

In addition to the selective recognition of the diphosphorylated peptides in water by mAbs AT10 and PHF-27 there are two further conclusions to be drawn from the data presented here:

(1) The binding of the studied anti-PHF antibodies to the phosphopeptides is strongly dependent upon the length and/or the purity of the antigens as well as the dilution of the antibody preparation. MAbs PHF-20 and PHF-1 recognized phosphorylated amino acids other than the main sites (Ser214 for PHF-20 and Ser396 for PHF-1) in antigen preparations when the antigens were longer (Otvos et al., 1994) or less homogenous. This fully explains the discrepancy found in the literature for not only peptide recognition studies (Zemlan & Dean, 1996), but also when the full τ protein was analyzed. Both fetal τ and biopsy-derived τ are known to be heterogeneously phosphorylated (Matsuo et al., 1994), so the controversial results may have arisen from the different experimental conditions of the production and purification of the different τ proteins.

(2) PHF-τ contains immunodominant phosphoserine and phosphothreonine residues in neighboring sites. The three most characteristic single sites are Ser214, Thr231, and Ser396 with participation of nearby phosphoamino acids (Thr212, Thr217, Ser235, and Ser404). Regardless of which laboratory generated them, the mAbs against these phosphoamino acids were always dominant and the mAbs showed the same specificity. This immunodominance can be explained on the basis of increased stability of these phosphorylated amino acids *in vivo*, as our serum stability *in vitro* indicates (Hoffmann et al., 1997). In turn, this suggests that the antibody repertoire that can be produced by using the full protein as immunogen is limited. Synthetic phospho-

peptides, which are much shorter than the protein and contain designed phosphoserines or phosphothreonines, display and present each of the individual phosphoamino acids and the multiphosphorylated neighboring sites much more efficiently than the long, heterogeneously phosphorylated protein does. Antibodies against designed phosphopeptides offer considerably increased variety and specificity to hidden linear or conformational epitopes that may be singly or, more likely, multiply phosphorylated in PHF-τ but not in normal adult τ, fetal τ, or biopsy-derived τ. Our preliminary data obtained after immunization with the τ 224−240 231P, 235P peptide supports this hypothesis.

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