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Abnormal phosphorylation of the microtubule-associated protein τ (tau) in Alzheimer cytoskeletal pathology

(Alzheimer disease/neurofibrillary tangles/paired-helical filaments/microtubules)

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ABSTRACT **A monoclonal antibody to the microtubule-associated protein τ (tau) labeled some neurofibrillary tangles and plaque neurites, the two major locations of paired-helical filaments (PHF), in Alzheimer disease brain. The antibody also labeled isolated PHF that had been repeatedly washed with $NaDodSO_4$. Dephosphorylation of the tissue sections with alkaline phosphatase prior to immunolabeling dramatically increased the number of tangles and plaques recognized by the antibody. The plaque core amyloid was not stained in either dephosphorylated or nondephosphorylated tissue sections. On immunoblots PHF polypeptides were labeled readily only when dephosphorylated. In contrast, a commercially available monoclonal antibody to a phosphorylated epitope of neurofilaments that labeled the tangles and the plaque neurites in tissue did not label any PHF polypeptides on immunoblots. The PHF polypeptides, labeled with the monoclonal antibody to τ, electrophoresed with those polypeptides recognized by antibodies to isolated PHF. The antibody to τ-labeled microtubules from normal human brains assembled *in vitro* but identically treated Alzheimer brain preparations had to be dephosphorylated to be completely recognized by this antibody. These findings suggest that τ in Alzheimer brain is an abnormally phosphorylated protein component of PHF.**

In Alzheimer disease/senile dementia of the Alzheimer type (AD/SDAT) the most characteristic neuropathological lesion is the accumulation of paired-helical filaments (PHF). These abnormal cytoskeletal fibrils accumulate as neurofibrillary tangles in neuronal cell bodies and in the neurites of the neuritic (senile) plaques. Each PHF is made up of eight protofilaments, 3–5 nm in diameter (1). The PHF protofilaments differ ultrastructurally from those of normal neurofibrils (2). Biochemical studies of the PHF have been hampered because of difficulties in their isolation and solubilization. A purification procedure for PHF has enabled partial characterization of component polypeptides by $NaDodSO_4$/PAGE (3). Subsequent studies with both polyclonal and monoclonal antibodies (mAb) to PHF have demonstrated that polypeptides from 45 to 62 kDa are PHF specific (4, 5). Since these PHF polypeptides were also labeled by antibodies to microtubule proteins, but not by antibodies to tubulin (5–7), it appeared possible that they might belong to the heterogeneous group of microtubule-associated proteins, called τ (tau) (8). Using an mAb to τ, the present study demonstrates that τ in Alzheimer brain is atypically phosphorylated and is a component of PHF.

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MATERIALS AND METHODS

Antibodies and Immunological Reagents. mAb tau-1 to τ was generated in mice using $NaDodSO_4$-denatured microtubule-associated proteins from bovine brain and affinity purified by protein A-Sepharose chromatography (9). The tau-1 antibody recognizes τ from calf, rat, and human brain, and the epitope recognized seems confined exclusively to τ (9, 10). In the experiments described below tau-1 was employed at a concentration of 0.1 μg/ml. Antiserum to isolated PHF (4) was diluted at 1:1000. PHF-reactive antiserum to calf microtubules (anti-MT II serum), antiserum to microtubules (anti-MT IV serum), and antiserum to neurofilaments have been described (4, 7). These three antisera were used for immunoblots at 1:3000, 1:1000, and 1:2000 dilutions, respectively. The two latter antisera do not react with PHF. Commercially available antibodies used were affinity purified antibodies to tubulin [CAABCO, Houston, TX; 1 μg/ml (7)]; mAb YL 1/2 to α tubulin (Accurate Chemicals, Westbury, NY; ascites, 1:100); mAb NR 4 to neurofilament polypeptide P68 (Boehringer Mannheim; 1 μg/ml); and mAbs SMI 31 and SMI 34 to phosphorylated epitopes in neurofilament polypeptides P150 and P200 (Sternberger–Meyer Immunocytochemicals, Jarrettsville, MD; ascites, 1:1000 to 1:15,000). Reagents for the avidin–biotin complex technique were purchased from Vector Laboratories (Burlingame, CA), goat anti-rabbit IgG serum was from Sternberger–Meyer, and peroxidase anti-peroxidase complex was from Cappel Laboratories (Cochranville, PA).

Immunocytochemistry. Monoclonal antibody to τ was tested by avidin–biotin complex technique (11) on 6-μm sections of formalin-fixed and paraffin-embedded hippocampus from six AD/SDAT patients ranging in age from 56 to 85 years (postmortem times, 3–24 hr), hippocampus from an 80-year-old non-Alzheimer dementia individual and cerebellum from one each of the above AD/SDAT patients and from a nondemented 70-year-old individual. Dephosphorylation of adjacent sections was carried out prior to application of antibodies (12) using either calf intestinal alkaline phosphatase alone (43 μg/ml or 130 μg/ml; type VII, Sigma; 0.1 M Tris·HCl, pH 8.0, containing 0.01 M phenylmethylsulfonyl fluoride at 32°C for 2.5 hr) or trypsin (400 μg/ml; GIBCO; at 37°C for 10 min) followed by incubation with alkaline phosphatase (130 μg/ml). In control experiments sections were incubated in either (*i*) above Tris buffer without enzymes, (*ii*) Tris buffer containing only trypsin, (*iii*) alkaline phosphatase

Abbreviations: AD/SDAT, Alzheimer disease/senile dementia of the Alzheimer type; PHF, paired-helical filaments; mAb, monoclonal antibody.

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at 43 μg/ml together with 250 mM phosphate, or (*iv*) 2.5 mM EDTA and 2.5 mM EGTA.

PHFs were isolated from the brain of a 72-year-old Alzheimer patient using the long procedure of Iqbal *et al.* (3). Microtubules were prepared from the cerebrum of an 83-year-old Alzheimer patient and from two normal individuals, 40 and 81 years old, by *in vitro* assembly (13).

Protein Determination, $NaDodSO_4$/PAGE and Immunoblots. Protein concentrations were estimated by the method of Lowry *et al.* (14) as modified (3) and verified by amino acid analysis and Coomassie blue-stained gels. $NaDodSO_4$/PAGE (80 × 60 × 0.75 mm slab) was performed according to Laemmli (15). Sample preparation was as described (3). The isolated PHF were solubilized by ultrasonication for 30 min (3) followed by heating in 1% each of $NaDodSO_4$ and 2-mercaptoethanol; over 90% of the PHF polypeptides are solubilized by this treatment. After electrophoresis PHF polypeptides and prestained protein standards (Bethesda Research Laboratories) were transferred to nitrocellulose (0.2 μm, Schleicher & Schuell) at 100 V for 1 hr using the buffer of Towbin *et al.* (16). Residual protein binding sites were blocked with 5% (wt/vol) defatted dry milk in 50 mM Tris·HCl, pH 7.6, containing 0.2 M NaCl for 2 hr at 22°C. The blots were then incubated for 18 hr with antibodies diluted in the blocking solution. Bound mAb were detected by using the avidin–biotin complex technique (5). Detection of polyclonal antibodies was by the peroxidase-antiperoxidase technique (17). For dephosphorylation experiments the blots were blocked in 20% (wt/vol) bovine serum albumin (crystalized, Sigma) in Tris buffer and incubated with alkaline phosphatase at 43 μg/ml (12).

RESULTS

Staining of tangles and plaque neurites by the mAb to τ was observed in hippocampus tissue sections from two of the six AD/SDAT patients (Fig. 1*a*). In the remaining four patients only rare tangles and plaques were immunolabeled (Fig. 1*d*). In contrast dephosphorylation (43 μg of alkaline phosphatase per ml) of the adjacent sections prior to labeling with mAb to τ changed the staining pattern dramatically: in all six AD/SDAT individuals studied apparently every tangle present, identified by Bodian silver stain of the neighboring section, was intensely stained by the antibody (Fig. 1 *b* and *e*). In some neurons darkly stained outlines of cytoplasmic vacuoles of granulovacuolar degeneration were observed (Fig. 1*g*), a lesion frequently present in the hippocampal pyramidal neurons in AD/SDAT. In some of the affected areas staining of innumerable short thread-like structures scattered throughout the neuropil was striking after dephosphorylation (Fig. 1*e*). Similar labeling of the neuropil has also been described with antisera raised against PHF (4). These neuropil threads have been shown to contain accumulations of PHF (18). Although the neurites of the plaques were darkly stained by anti-τ antibody, immunolabeling of their amyloid cores was not seen (Fig. 1*c*). No change in staining pattern or intensity was observed with increased phosphatase concentration (130 μg/ml) or when trypsin digestion preceded the phosphatase treatment. However, when the sections were incubated with alkaline phosphatase in the presence of 2.5 mM each of EDTA and EGTA or 250 mM phosphate, inhibitors of enzyme activity, only faint immunostaining was observed. No staining with mAb to τ was seen in cerebellar sections of Alzheimer or normal brain under any of the conditions tested (figure not shown). Lack of immunostaining of normal axons with mAb to τ suggests that the antigenicity of normal τ was lost in the formalin-fixed paraffin-embedded tissue. In the non-Alzheimer dementia individual no immunolabeling of any cellular component was observed in sections of hippocampus except for an occasional tangle and a few neuropil threads (Fig. 1*h*). Postmortem intervals up to 24 hr did not appear to affect the immunostaining results.

PHF are surrounded by a coat of amorphous material that disappears after short exposure of a tangle-enriched fraction to 2% (wt/vol) $NaDodSO_4$ at room temperature (3). To study whether the antigen recognized by anti-τ antibody might be a contaminant, a tangle-enriched fraction was prepared under nondenaturing conditions (3) and treated with 2% (wt/vol) $NaDodSO_4$ for 5 min at room temperature. Twice the material was pelleted and heated with 2% (wt/vol) $NaDodSO_4$ in a boiling water bath for 5 min. Significant numbers of tangles were lost by this treatment. Immunolabeling of samples (11) taken after each of these detergent treatments showed that the antibody bound to tangles isolated under nondenaturing conditions as well as tangles that had been washed extensively with $NaDodSO_4$ (Fig. 1*i*). These findings indicated that τ was intrinsic to PHF.

Only very faint immunolabeling of the PHF polypeptides by anti-τ antibody was seen on immunoblots of the PHF preparation (Figs. 2 and 3). However, when the blots were treated with alkaline phosphatase (43 μg/ml) prior to application of the antibody, 4 or 5 polypeptide bands with M_r ranging from about 55,000 to 62,000 were distinctly labeled (Figs. 2 and 3). In addition, immunolabeling of material at the top of spacer and resolving gels, a smear along most of the lane, and several polypeptides of M_r below 45,000 was also observed. A comparison of PHF polypeptides labeled with PHF-reactive anti-microtubule serum and with antiserum to isolated PHF revealed that the major polypeptides labeled by these antibodies had the same electrophoretic mobilities as the 55- to 62-kDa polypeptides labeled by mAb to τ (Fig. 2); both of these antisera have been shown to label the same PHF polypeptides as the mAb to isolated PHF (5). In contrast to the mAb to τ, the PHF-reactive anti-microtubule serum and the monoclonal and polyclonal antibodies to PHF recognized both phosphorylated and dephosphorylated forms of PHF polypeptides (data not shown).

PHF isolated by the procedure used in this study are highly purified (3), and on immunoblots tubulin, high molecular weight microtubule-associated proteins, or neurofilament triplet polypeptides were not detected using the following antibodies: affinity-isolated antibodies to tubulin; antiserum to calf microtubules; antiserum to neurofilaments; mAb to neurofilament polypeptide P68; and mAb SMI 31 and 34 to phosphorylated epitopes shared by neurofilament polypeptides P200 and P150. One of these antibodies, SMI 34, labels Alzheimer neurofibrillary tangles in tissue sections because of a phosphorylated epitope shared between neurofilaments and PHF. On immunoblots, however, this antibody only labeled the neurofilament (Fig. 2, lane 4, NF) not the PHF polypeptides (Fig. 2, lane 4, PHF). These results suggest that the reactivity of SMI 34 with PHF in tissue sections is most likely due to an assembled topographic rather than a sequential epitope. However, the possibility that the epitope consists of a continuous sequence of amino acids and was lost during the isolation of PHF cannot be eliminated.

The PHF polypeptide bands labeled by mAb to τ had the same electrophoretic mobilities as the τ polypeptides in the microtubule protein preparations (Fig. 3). However, like τ in PHF, τ in the Alzheimer microtubule fraction had to be dephosphorylated to be fully recognized by the τ mAb. The immunostaining of the τ polypeptides in the Alzheimer microtubule preparation, especially the two slowest moving molecular species, was enhanced by dephosphorylation (Fig. 3, lane B*), whereas dephosphorylation did not significantly change the staining of τ in microtubules from normal individuals studied (Fig. 3, lanes C).

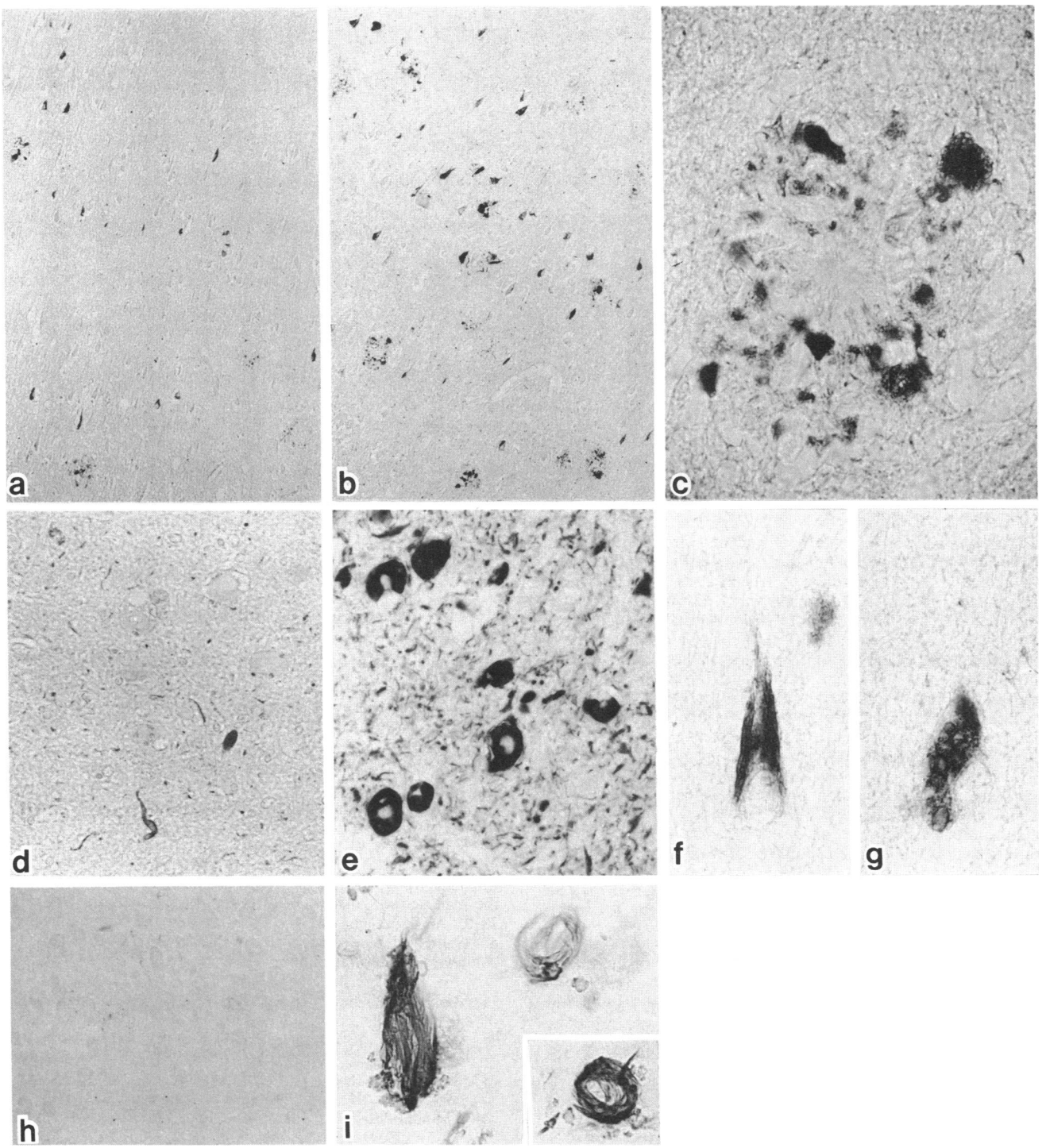

FIG. 1. Immunocytochemical staining with mAb to τ and the effect of dephosphorylation. (*a–c, f, g*) Sections of Alzheimer hippocampus and (*d, e*) temporal cortex; (*h*) section of hippocampus of a 80-year-old non-Alzheimer dementia individual; (*i*) tangle-enriched preparation that had been washed twice with 2% (wt/vol) $NaDodSO_4$ in a boiling water bath. (*b, c, e–h*) Sections were dephosphorylated with alkaline phosphatase prior to immunolabeling; (*a* and *d*) nondephosphorylated controls; adjacent sections and corresponding areas to *b* and *e* treated identically except that the alkaline phosphatase was substituted with buffer. Numbers of immunostained tangles, plaques, and neuropil threads are very much increased in the dephosphorylated tissue sections in *b* and *e* as compared to the control treated sections in *a* and *d*. (*c*) Staining of plaque neurites but not of central core amyloid; (*f*) a neuron with immunolabeled tangle extending into the apical dendrite; (*g*) a neuron with granulovacuolar inclusions; (*h*) no staining is seen in the non-Alzheimer hippocampus even after dephosphorylation. [(*a, b*) ×75; (*c, f, g*, and *i*) ×750; (*d, e*) ×300; (*h*) ×150.]

DISCUSSION

This study (*i*) demonstrates the similarity of τ and the major PHF polypeptides by their immunological crossreactivity and similar mobilities in $NaDodSO_4$/PAGE and (*ii*) shows that τ in Alzheimer brain is abnormally phosphorylated. This phosphorylation appears to make a highly conserved τ epitope inaccessible to a mAb to τ. These data suggest that phosphorylation of τ might be a factor in the formation of the PHF. Such a defect in AD/SDAT might be caused by an inbalance of the protein kinase–phosphatase system, involving either (*i*) increase of a certain protein kinase activity or (*ii*) reduction in the level of phosphatase activity that normally would control phosphorylation.

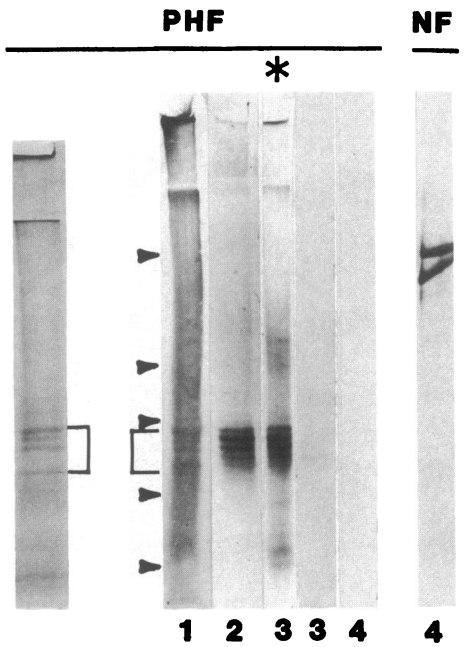

FIG. 2. Immunoblots of PHF polypeptides with (lane 1) antiserum to isolated PHF, 1:1000 dilution; (lane 2) PHF-reactive anti-microtubule serum, 1:3000 dilution; (lane 3) mAb to τ at 0.1 μg/ml on dephosphorylated (*) and nondephosphorylated blots and (lanes 4) blots of PHF and neurofilament (NF) polypeptides with mAb to NF, SMI 34, 1:10,000 dilution. (*) The dephosphorylation of PHF polypeptides on the paper blots was carried out with alkaline phosphatase (43 μg/ml) before incubation with antibody. Proteins were electrotransferred from NaDodSO$_4$/polyacrylamide gel, 5–15% acrylamide gradient. Arrowheads indicate positions of M_r markers from top to bottom: myosin (200,000), phosphorylase *b* (92,500), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700). Not shown in this figure, even at a 10-fold increase in the antibody concentration mAb SMI 34 did not label PHF polypeptides. The background smear and the low M_r bands in lane 1 most probably represent oligomers and breakdown products, respectively, of the PHF polypeptides (3–5); similar immunostaining pattern is obtained with mAb to PHF (5). The far left lane shows the Coomassie blue-stained polypeptide pattern of isolated PHF (5–30% acrylamide gradient).

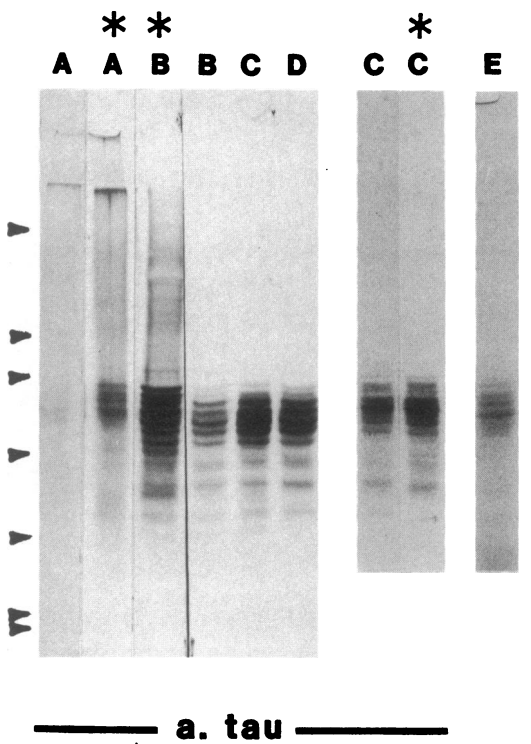

FIG. 3. Immunoblots with mAb to τ of PHF (lane A) and brain microtubule fractions (lanes B–D) from (lane B) 83-year-old Alzheimer patient, and 40-year-old (lane C) and 81-year-old normal individual (lane D) dephosphorylated (*) or untreated with alkaline phosphatase prior to incubation with the antibody. Proteins were electrotransferred from NaDodSO$_4$/polyacrylamide gel, 7–10% acrylamide gradient. Arrowheads indicate positions of M_r markers as in Fig. 2 and in addition β-lactoglobulin (18,400) and cytochrome *c* (12,300). The minor τ bands below M_r 45,000 are most likely the degradative products of the 55–62 kDa parent τ polypeptides. Coomassie blue-stained patterns of τ purified from calf brain (10) (lane E).

Protein phosphorylation is believed to be one of the major mechanisms for regulation of cellular function. τ is a phosphoprotein (19). It was discovered and is best known for promoting the *in vitro* assembly of microtubules (8). The interaction of τ with other brain proteins appears to be regulated by phosphorylation. The *in vitro* phosphorylation of τ has been reported to inhibit both the polymerization of tubulin (20) and the interaction of microtubules with actin filaments (19). Furthermore, preliminary data suggest a defect in microtubule assembly in AD/SDAT (21). The abnormal phosphorylation of τ observed in AD/SDAT brain might depress its normal interaction with other brain proteins and could also lead to the assembly of τ, either alone or with other neuronal components, into PHF. The abnormal phosphorylation of τ in vulnerable neurons in AD/SDAT might take place over a long period of time. Some tangles, for instance those recognized by the mAb to τ without prior dephosphorylation, might represent earlier stages in this process than those recognized only after dephosphorylation.

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