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# Implication of brain cdc2 and MAP2 kinases in the phosphorylation of tau protein in Alzheimer's disease

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Brain tau protein is phosphorylated in vitro by cdc2 and MAP2 kinases, obtained through immunoaffinity purification from rat brain extracts. The phosphorylation sites are located on the tau molecule both upstream and downstream of the tubulin-binding motifs. A synthetic peptide comprising residues 194–213 of the tau sequence, which contains the epitope recognized by the monoclonal antibody tau-1, is also efficiently phosphorylated in vitro by cdc2 and MAP2 kinases. Phosphorylation of this peptide markedly reduces its interaction with the antibody tau-1, as it has been described for tau protein in Alzheimer's disease. Both cdc2 and MAP2 kinases are present in brain extracts obtained from Alzheimer's disease patients. Interestingly, the level of cdc2 kinase may be increased in patient brains as compared with non-demented controls. These results suggest a role for cdc2 and MAP2 kinases in phosphorylating tau protein at the tau-1 epitope in Alzheimer's disease.

Tau protein; Microtubule-associated protein; Proline-directed protein kinase; Alzheimer's disease

## 1. INTRODUCTION

Senile dementia of Alzheimer's type (AD) is characterized by the massive accumulation of aberrant structures including neurofibrillary tangles (NFTs) in the patient's brains [1]. NFTs are derived from intraneuronal inclusions consisting of aggregated paired helical filaments (PHFs), which contain as a major core component a modified form of the microtubule-associated tau protein [2]. This PHF tau is less soluble, higher in molecular weight and more acidic than normal microtubule-associated tau [3–5]. PHF tau also displays immunoreactivity for certain antibodies recognizing phosphorylated epitopes [5–11] and shows a diminished binding to the monoclonal antibody tau-1, which reacts with a phosphorylation-sensitive epitope [12]. These distinctive features of PHF tau are due to its abnormally hyperphosphorylated state with respect to that of normal tau [2–15].

The major role for tau protein in normal neurons may be the stabilization of microtubules [16,17], mainly within axons [18,19]. The binding of tau to microtubules is thought to be modulated by tau phosphorylation [20]. Hyperphosphorylation of tau protein might therefore cause the disorganization of the microtubule cytoskeleton, blocking axonal transport, in addition to allow the deposition of hyperphosphorylated tau into PHFs, thus leading to neurodegeneration. Supportive of this view is the fact that tau hyperphosphorylation precedes NFT formation in neurons showing the earliest degenerative changes [21,22].

Thus, there is a great interest in elucidating the protein kinases implicated in tau hyperphosphorylation in AD. An important clue is provided by the fact that several Ser/Thr–Pro sequences on tau protein are found phosphorylated in PHF tau [4,10,23]. Likewise, the phosphorylation of tau protein by proline-directed protein kinase (PDPK) activities present in cell extracts [24], brain cytosol [25,26] and in brain cytoskeletal [27] and tau [28–30] preparations has been demonstrated. In this report we analyze the phosphorylation of tau, mainly at the tau-1 epitope, by two well-known PDPKs, cdc2 and MAP2 kinases, which were isolated from rat brain cytosol, and examine their presence in extracts from AD patient brains.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Monoclonal antibody tau-1 [18,31] was a generous gift of Dr. L.I. Binder (University of Alabama). Polyclonal antibodies against the C-terminus of p34 cdc2 [32,33] were gifts of Dr. P. Nurse (University of Oxford) and Dr. G. Draetta (EMBL, Heidelberg). A polyclonal antibody against the conserved PSTAIR peptide, present in cdc2 and cdc2-like kinases [34], was purchased from UBI (N.Y., USA). A monoclonal antibody to MAP2 kinase [35] was purchased from ZYMED Laboratories Inc. (San Francisco, CA, USA).

The peptides RSGYSSPGSPGTPGSRSRTP (comprising residues 194–213 of the tau sequence), YSSPGSPGTP (comprising residues 197–206 of the tau sequence), and GTPGSRSR (comprising residues 204–211 of the tau sequence) were synthesized on an automatic solid phase peptide synthesizer (type 430 A, Applied Biosystems) and purified by reverse-phase HPLC on a Nova Pak C18 column. Peptides were coupled with bovine serum albumin (BSA) using glutardialdehyde as cross-linker.

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2.2. *Protein preparation*

Tau was purified from bovine brain microtubules according to the procedure of Herzog and Weber [36].

2.3. *Protein kinases*

Protein kinases were obtained through immuno-adsorption essentially as described by Boulton and Cobb [37]. The antibody to the C-terminus of p34 cdc2 provided by Dr. Nurse was used to prepare cdc2 kinase from 5-day-old rat pup brain extracts. Extracts from adult rat brain were used as a source to prepare MAP2 kinase. Protein kinases were assayed using 50 $\mu$M [$\gamma$-$^{32}$P]ATP and calf thymus histone H1 or rat brain MAP2 as substrates.

2.4. *Protein and peptide phosphorylation*

The phosphorylation of tau (20 $\mu$g) with either cdc2 or MAP2 kinases was performed for 1 h at 37°C in 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 5 mM $MgCl_2$ and 50 $\mu$M [$\gamma$-$^{32}$P]ATP. Phosphorylation reactions were stopped by the addition of boiling SDS sample buffer.

Phosphorylation of the synthetic peptides was carried out under the conditions described for tau phosphorylation, except that 3 h of incubation at 37°C was used. Phosphorylated peptide was separated from ATP by reverse-phase chromatography on a Nova Pack C18 column equilibrated with 0.1% trifluoroacetic acid in water and eluted using a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid.

The phosphorylation of the synthetic peptide conjugated with BSA was performed for 3 h at 37°C in 50 mM Tris-HCl, pH 7.5; 2 mM EGTA, 5 mM $MgCl_2$ and 5 mM ATP. Phosphorylation was stopped by the addition of boiling SDS sample buffer.

2.5. *Gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli [38]. Phospholabeled proteins were detected by autoradiography of dried gels exposed to Kodak-X-Omat films.

2.6. *Cysteine-specific chemical cleavage of tau protein*

Phosphorylated tau protein bands were excised from the gels, equilibrated in 0.1 M Tris-HCl, pH 8.0, methanol (50% v/v) and treated with 2 mM 2-nitro-5-thiocyanobenzoic acid (NTCB) dissolved in 7.5 M urea, 0.2 M Tris-HCl, 1 mM EDTA, pH 8.0, as described before [27].

2.7. *Phosphoamino acid analysis*

Phosphorylated peptides were hydrolyzed with 6 N HCl for 4 h at 110°C. Phosphoamino acids were separated by one-dimensional electrophoresis on cellulose thin layer using acetic acid/pyridine/water (50:5:945) at 900 V for 60 min.

2.8. *Immunoblotting assays*

Samples were either electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose paper or directly dotted onto nitrocellulose paper and probed with antibodies. Immunoreactive proteins were visualized by the use of peroxidase-conjugated or alkaline phosphatase-conjugated secondary antibodies.

2.9. *Human brain extracts*

Samples from temporal cortex gray matter from brains from three AD or age-matched non-demented subjects were collected after short post-mortem periods and frozen in liquid nitrogen. To prepare the extracts, samples were thawed, homogenized in 50 mM Tris-HCl pH 7.5; 2% SDS and centrifuged at 100,000 × $g$ for 1 h. Aliquots of the supernatants containing the same amount of protein were characterized by gel electrophoresis and immunoblotting.

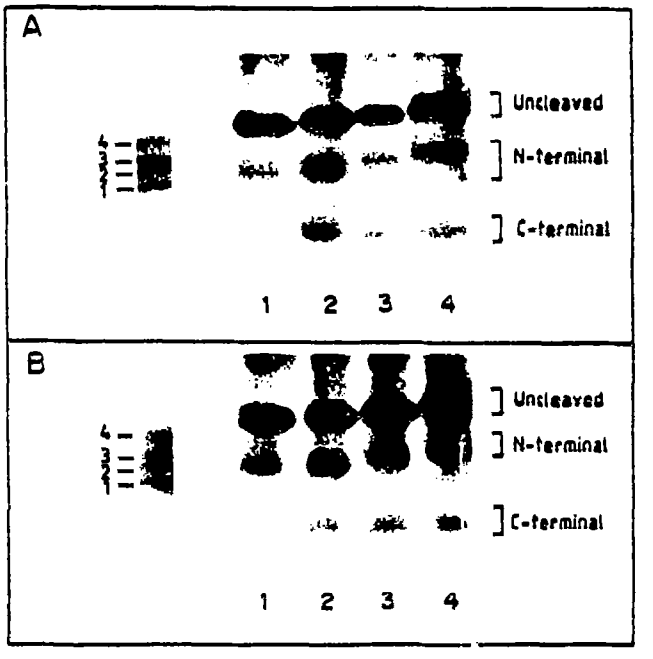


Fig. 1. Phosphorylation of tau protein by cdc2 and MAP2 kinases. Bovine brain tau was phosphorylated in vitro by either cdc2 or MAP2 kinase and subjected to SDS-gel electrophoresis and autoradiography. (A) The autoradiogram shows tau protein bands phosphorylated by cdc2 kinase and numbered in order of increasing molecular weight (left). The NTCB phosphopeptide maps of these tau protein bands are shown in the autoradiography to the right. The positions of uncleaved tau, and of the N-terminal and C-terminal fragments are indicated. (B) The autoradiogram shows tau protein band phosphorylated by MAP2 kinase (left). The corresponding NTCB phosphopeptide maps of these tau protein bands are shown to the right.

## 3. RESULTS

### 3.1. *Phosphorylation of tau protein by cdc2 and MAP2 kinases*

Both cdc2 and MAP2 kinases, obtained through immunoaffinity purification from rat brain extracts, phosphorylate bovine brain tau protein. In our electrophoretic system, bovine brain tau is resolved into four intense bands, all of which are phosphorylated by these protein kinases (Fig. 1). These results are consistent with the previously reported phosphorylation of tau by a PDPK present in rat brain cytoskeletal protein preparations obtained in our laboratory [27]. This PDPK actually corresponds to MAP2 kinase, which is associated with microtubules (unpublished results).

The distribution of phosphorylation sites on tau molecule was studied after cysteine-specific chemical cleavage by *S*-cyanylation using NTCB. The NTCB phosphopeptide maps obtained for tau protein phosphorylated by cdc2 or MAP2 kinases show that both amino- and carboxy-terminal fragments are phosphorylated (Fig. 1). Densitometric scanning of the corresponding autoradiographies suggests the presence of more than three phosphorylation sites on the amino-

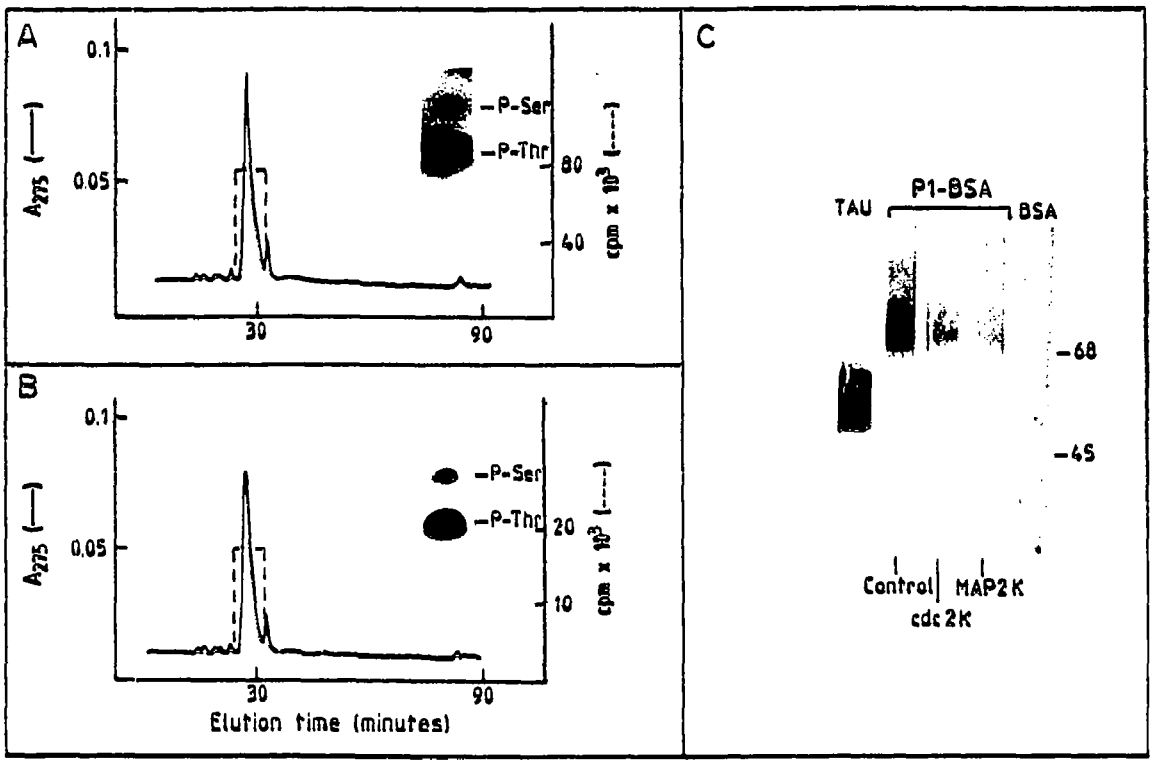


Fig. 2. Masking of the tau-1 epitope after phosphorylation by cdc2 and MAP2 kinases. (A) The synthetic peptide P1 bearing the tau-1 epitope was phosphorylated by cdc2 kinase and purified by HPLC. Absorbance at 275 nm of the eluted peptide is shown (——) together with the radioactivity (---) associated with the peptide peak. Inset shows the phosphoamino acid analysis of the phosphorylated peptide. (B) The synthetic peptide P1 bearing the tau-1 epitope was phosphorylated by MAP2 kinase and analyzed as described in A. (C) Immunoblotting assay showing the masking of tau-1 epitope after phosphorylation. Aliquots (20 $\mu$g) of bovine brain tau protein (TAU), unphosphorylated peptide P1 coupled with BSA (P1-BSA, control), peptide P1 coupled with BSA and in vitro phosphorylated by cdc2 kinase (P1-BSA, cdc2 K) peptide P1 coupled with BSA and in vitro phosphorylated by MAP2 kinase (P1-BSA, MAP2 K) and BSA (BSA) were probed with monoclonal antibody tau-1 after Western blotting. Numbers to the right indicate the position of molecular weight markers in kDa.

terminal fragment per phosphorylation site on the carboxy-terminal fragment. We have previously demonstrated that a synthetic peptide corresponding to a tubulin-binding motif is not phosphorylated by PDPK [27]. Thus, phosphorylation sites for cdc2 and MAP2 kinases must be located both upstream and downstream of the repeated tubulin-binding motifs on tau molecule. This is consistent with the presence of putative Ser/Thr–Pro targets for cdc2 and MAP2 kinases on tau. There are four Ser–Pro and ten Thr–Pro putative targets on the amino-terminal fragment, whereas there are only three Ser–Pro putative targets on the carboxy-terminal fragment of tau.

Of particular relevance is the clustering of most putative phosphorylation targets at a proline-rich region immediately upstream of the tubulin-binding motifs, as the conformation of this region, also present in MAP2 and MAP4, is thought to affect tubulin-binding [39].

### 3.2. *Phosphorylation of a synthetic peptide bearing the tau-1 epitope by cdc2 and MAP2 kinases*

The epitope for the monoclonal antibody tau-1, which is masked upon phosphorylation in AD [12], is approximately located within the Pro-rich region of tau molecule [40]. We have determined that a synthetic peptide (P1 = RSGYSSPGSPGTPGSRSRTP) comprising residues 194–213 of the tau sequence bears the tau-1 epitope, as it is recognized by the monoclonal antibody tau-1 in dot-blot assays and it totally competes with tau protein for antibody tau-1 binding. Shorter synthetic peptides (P2 = YSSPGSPGTP; P3 = GTPGSRSR) are weakly recognized by tau-1 in dot-blot assays but are practically unable to compete with tau protein for tau-1 binding (not shown), which suggests an influence of the conformation of the peptide in its immunoreactivity against the antibody tau-1.

The synthetic peptide bearing the tau-1 epitope (peptide P1) is efficiently phosphorylated by both cdc2 and MAP2 kinases (Fig. 2). Phosphorylation mainly occurs at threonine, although some serine is also modified (Fig. 2 insets).

Peptide P1 coupled with bovine serum albumin (P1-BSA) is also efficiently phosphorylated by both cdc2 and MAP2 kinases. Interestingly, the peptide P1 coupled with BSA reacts with antibody tau-1 in immunoblot assay, as expected, and this reaction is almost

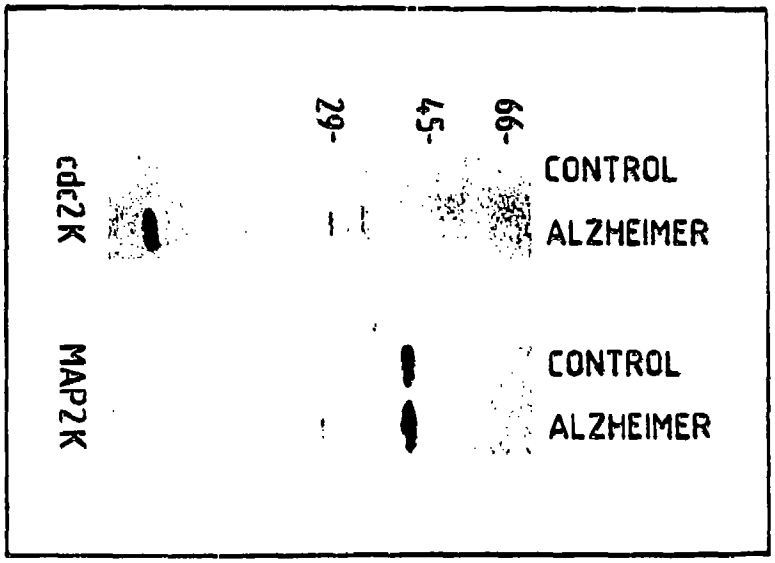


Fig. 3. Presence of cdc2 and MAP2 kinases in human brain extracts. Brain extracts from age-matched AD patients and control non-demented subjects were obtained as described in section 2 and probed with antibodies to cdc2 kinase (cdc2 K) and MAP2 kinase (MAP2 K). Numbers to the left indicate the positions of molecular weight markers in kDa.

abolished after phosphorylation by either cdc2 or MAP2 kinases (Fig. 2). Thus, both cdc2 and MAP2 kinases are able to phosphorylate and mask the tau-1 epitope.

### 3.3. *Presence of cdc2 and MAP2 kinases in AD brain*

Because of their possible implication in phosphorylating the tau-1 epitope, we have tested for the presence of cdc2 and MAP2 kinases in adult human brain extracts from control non-demented and AD patients. Fig. 3 shows that both kinases are present in AD brain extracts subjected to SDS-PAGE and immunoblotting. Whereas the level of MAP2 kinase is high and similar in AD and control human brain, cdc2 kinase, which does not seem to be as abundant as MAP2 kinase, is more prominent in AD brain than in control human brain (Fig. 3). This cdc2 kinase is recognized by three different antibodies, two of them raised against the C-terminus of p34 cdc2 (the generous gifts from Dr. Nurse and Dr. Draetta) and a third raised against the synthetic peptide PSTAIR. Fig. 3 shows the immunoblotting with one of the antibodies to the C terminus of p34 cdc2.

## 4. DISCUSSION

Cdc2 and MAP2 kinases are thought to play major roles in regulating cell growth and differentiation through the phosphorylation of a variety of proteins which may include components of the cytoskeleton [41–43].

Thus, different forms of cdc2 kinase have been implicated in the control of DNA replication and mitosis in proliferating cells [41,42]. In particular, the mitotic form of cdc2 may regulate the formation of the mitotic spindle [44], possibly through the phosphorylation of certain MAPs including MAP1B and MAP4 [45,46]. However, cdc2 kinase is also present in differentiated PC12 cells exhibiting a neuronal-like phenotype [33] and in young postmitotic cerebellar neurons at the time of neurite outgrowth [47]. Terminal differentiation of neurons is accompanied by down-regulation of cdc2 [47]. This suggests a role for cdc2 during the early stages of neuronal morphogenesis.

There are also different forms of MAP2 kinase, referred to as ERKs (extracellular regulated kinases), which are ERK1 (p44 MAPK), ERK2 (p42 MAPK), ERK3 and ERK4 [43]. Similarly to cdc2, ERKs may also participate into mitotic regulation [48], presumably acting on MAPs [46,49]. In contrast to cdc2, the level of ERKs increases during rat brain development [50]. The activity of neuronal MAP2 kinase can be regulated in response to certain neurotrophic factors [50,51] and neurotransmitters [52]. It is plausible that MAP2 kinase is the main PDPK present in adult mammalian brain. The relationship, if any, of either cdc2 or MAP2 kinases with other PDPKs detected in brain [25–30] has not been addressed.

Our results show that cdc2 and MAP2 kinases, isolated from rat brain extracts, may phosphorylate tau proteins at the tau-1 epitope and that those kinases are present in AD brain. MAP2 and MAP4, two proteins belonging to the same MAP superfamily as tau [39], have also been described as in vitro substrates for both cdc2 [45,53,54] and MAP2 kinases [49,55]. It has been shown that the substrate specificities of cdc2 and MAP2 kinases partially overlap in vitro and that, at least in the

case of nuclear lamin, phosphorylation occurs at a common site [56]. This suggests the possibility of cooperation between cdc2 and MAP2 kinases in phosphorylating common substrates, which may be particularly important to amplify the degree of phosphorylation of abundant cytoskeletal proteins. However, it still remains unclear the extent of the overlap in substrate specificities between cdc2 and MAP2 kinases in vivo. An important factor in this respect could be the modulation of the substrate specificity displayed by the catalytic p34 cdc2 in response to its binding to cyclins which act as regulatory subunits [41,57]. There are several types of cyclins, referred to as A, B, C, D and E [58], but their precise influence on the substrate specificity of p34 cdc2 is not fully understood. On the other hand, nothing is known about the cyclin subunits which predominate in brain and are complexed with neuronal p34 cdc2 and related kinases.

A point of major concern is the regulation of tau phosphorylation at the tau-1 epitope in vivo, both under physiological conditions and in AD. In adult rat brain, tau protein dephosphorylated at the tau-1 epitope is confined to axons, whereas tau protein phosphorylated at the tau-1 epitope is present in neuronal cell bodies and dendrites [31]. If we assume that MAP2 kinase is the main PDPK able to phosphorylate the tau-1 epitope in adult rat brain, this would suggest a predominantly somatodendritic compartmentation of the enzyme. Curiously, the main in vitro substrate for MAP2 kinase is MAP2, which shows a strict somatodendritic compartmentation [59–61]. This might indicate a putative interaction between MAP2 and the kinase, which is supported by the fact that MAP2 kinase is indeed present in microtubule preparations (unpublished results).

In AD, most tau becomes phosphorylated at the tau-1 epitope [12]. This may be a consequence of a failure in the phosphatases acting at the tau-1 site, an over-activation of MAP2 kinases and/or an up-regulation of cdc2 kinases. Our results showing the presence of cdc2 and MAP2 kinases in AD brain support the latter possibilities. The up-regulation of cdc2 kinase does not seem to be unique, as a re-expression of certain proteins characteristic of the fetal brain after ageing, and particularly in AD brain, has been previously described [62,63]. This may be hypothetically connected with the massive and aberrant neurite regeneration supposedly associated with AD [63].

Clearly further research on the signal transduction mechanisms controlling the expression and activation of cdc2 and MAP2 kinases in neurons is required before understanding their alterations in AD. Some in vitro and animal models may be useful in this respect. For instance, certain PKC activators, which also enhance MAP2 kinase activity in cultured cells [64], trigger the appearance of immunoreactivity for antibodies recognizing PHF-tau in cultured human cortical neurons [65]. Likewise, heat shock, which induces a form of cdc2 kinase in cultured cells [66], also results in the hyperphosphorylation of tau protein at the tau-1 epitope in rat brain [67].

Finally, the functional consequences of tau phosphorylation at the tau-1 epitope should be considered. Phosphorylation of the Pro-rich region of tau molecule may diminish its binding to tubulin, as it has been reported for other MAPs, such as MAP2 and MAP4 [49]. This might be one of the events leading to the reduction in tubulin binding-competent tau and the subsequent disorganization of the microtubule cytoskeleton which are observed in AD.

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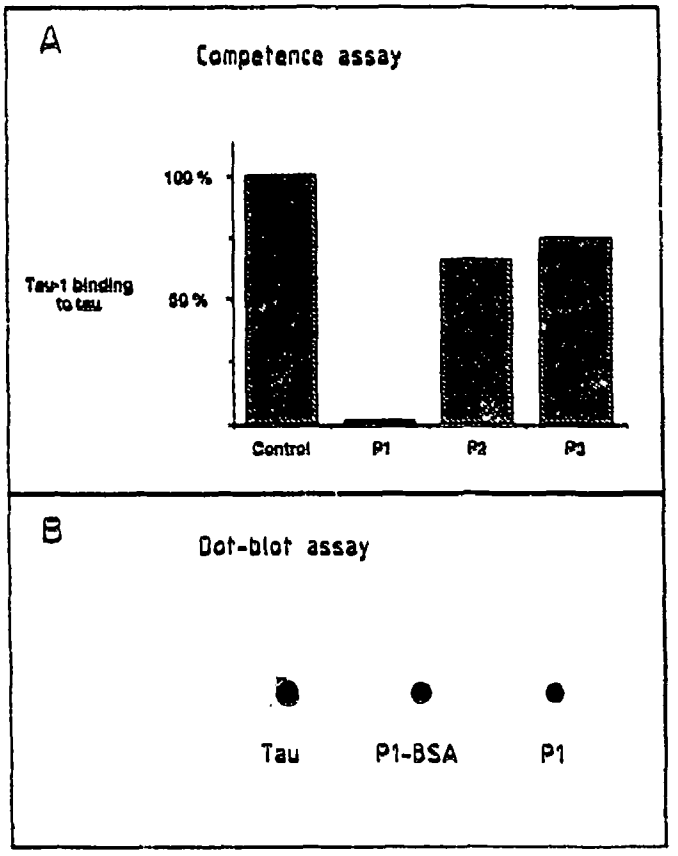
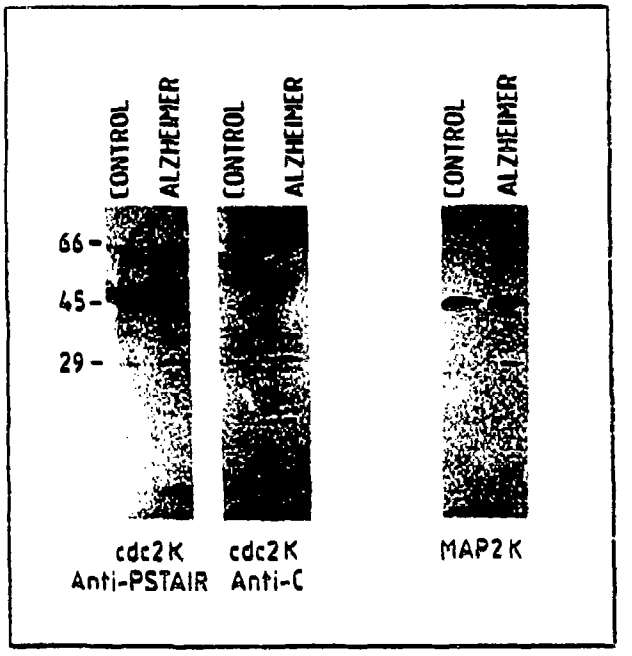
## APPENDIX




Appendix 1. Binding of synthetic peptides to monoclonal antibody tau-1. A. Competence assay between tau protein and synthetic peptides for tau-1 binding. Samples of 4 $\mu$M peptides P1, P2 and P3 were incubated with antibody tau-1 diluted 1:10,000 overnight. Then these samples were incubated with 1 $\mu$g of tau protein previously dotted onto nitrocellulose paper. Densitometric data are represented as histograms. In the control experiment, antibody tau-1 without any peptide was used. B. Dot-blot assay showing the binding of synthetic peptide P1 to antibody tau-1. Antibody tau-1 diluted 1:5,000 was probed with 0.3 $\mu$g of tau protein, peptide P1 coupled with BSA and peptide P1 alone dotted onto nitrocellulose paper.




Appendix 2. Presence of cdc2 and MAP2 kinases in human brain extracts. Brain extracts from age-matched AD patients and control non-demented subjects were obtained as described in section 2 and subjected to gel electrophoresis and immunoblotting. Blots were probed with two antibodies to cdc2 kinase (cdc2 K), one recognizing a common sequence to cdc2 and cdc2-like kinases (anti-PSTAIR) and another recognizing the C terminus of cdc2 kinase (anti-C), and one antibody to MAP2 kinase (MAP2 K).