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MITOSIS RELATED PHOSPHORYLATION OF THE NEURONAL MICROTUBULE-ASSOCIATED PROTEIN TAU

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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1998

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ABSTRACT

Alzheimer's disease (AD) is clinically characterized by the presence of dementia and loss of cognitive functions. Over time certain regions of the brain, primarily those involved in learning and memory, become damaged and deteriorate leading to the observed clinical symptoms. The primary neuropathological lesions associated with AD are senile plaques and neurofibrillary tangles (NFT). The major components of NFTs are the paired helical filaments (PHFs), which are formed from the self-assembly of the microtubule-associated protein tau. PHF-tau is abnormally phosphorylated, and this hyperphosphorylation is thought to contribute to the formation of NFTs. Several phosphorylation sites are shared between fetal- and PHF-tau suggesting that fetal-like or mitotic kinases might be reactivated in AD. Several Ser/Thr kinases, including cdc2 and cdk5, can phosphorylate tau on sites associated with AD. The focus of this research was to determine if the MPM2 antibody, which recognizes a Ser/Thr phosphoepitope present on a subset of mitotic proteins, reacted with hyperphosphorylated tau. Hippocampal AD brain sections were stained with MPM2 and PHF-tau antibody, AT8. Both antibodies recognized and colocalized to senile plaques and NFTs in the AD samples. PHFs isolated from AD brains reacted with both antibodies on immunoblots; however, normal fetal and
adult rat tau were not recognized by the MPM2 antibody. In vitro phosphorylation of recombinant human tau revealed that the MPM2 epitope was present on all tau isoforms, and could be phosphorylated by kinases present in both fetal and adult brain. The in vitro phosphorylation of tau also suggested a temporal sequence of phosphorylation, with the MPM2 epitope site being phosphorylated after other known PHF-tau sites, but prior to the AT8 site. Moreover, the MPM2 and AT8 sites on tau appeared to be distinct and regulated by different kinase and phosphatase activities. Phosphorylation of tau deletion mutants suggested that the MPM2 epitope was either located between amino acids 210-237 or within the C-terminus of the protein. Taken together, these results established that the MPM2 mitosis-associated phosphoepitope was present on PHF-tau, could be generated in vitro by kinases present in normal brain, and may be specific for the disease state.
Dedicated to my entire family

especially my parents, grandparents, and sister who always encouraged me to follow my dreams and my husband who keeps me going
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# TABLE OF CONTENTS

| ABSTRACT  | ii  |
| DEDICATION | iv  |
| ACKNOWLEDGEMENTS | v  |
| VITA       | vii |
| LIST OF FIGURES | xii |
| LIST OF ABBREVIATIONS | xv |
| INTRODUCTION | 1  |
| FIGURES    | 34  |

## CHAPTERS:

1. **ALZHEIMER'S DISEASE NEUROFIBRILLARY TANGLES CONTAIN MITOSIS SPECIFIC PHOSPHOEPITOPES**
   - INTRODUCTION ................................................................. 44
   - EXPERIMENTAL PROCEDURES............................................. 48
   - RESULTS ............................................................................ 54
   - DISCUSSION ....................................................................... 64
   - FIGURES ............................................................................ 70

2. **GENERATION AND CHARACTERISTICS OF TAU CONTAINING THE MITOSIS- AND ALZHEIMER'S DISEASE-ASSOCIATED MPM2 PHOSPHOEPITOPE FOLLOWING IN VITRO PHOSPHORYLATION**
   - INTRODUCTION ................................................................. 90
   - EXPERIMENTAL PROCEDURE ............................................... 95
   - RESULTS ............................................................................ 101
   - DISCUSSION ....................................................................... 110
   - FIGURES ............................................................................ 118
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>34</td>
</tr>
<tr>
<td>2.</td>
<td>35</td>
</tr>
<tr>
<td>3.</td>
<td>36</td>
</tr>
<tr>
<td>4.</td>
<td>37</td>
</tr>
<tr>
<td>5.</td>
<td>38</td>
</tr>
<tr>
<td>6.</td>
<td>39</td>
</tr>
<tr>
<td>7.</td>
<td>40</td>
</tr>
<tr>
<td>8.</td>
<td>41</td>
</tr>
<tr>
<td>9.</td>
<td>42</td>
</tr>
<tr>
<td>10.</td>
<td>43</td>
</tr>
<tr>
<td>11.</td>
<td>70</td>
</tr>
<tr>
<td>12.</td>
<td>72</td>
</tr>
<tr>
<td>13.</td>
<td>74</td>
</tr>
<tr>
<td>14.</td>
<td>75</td>
</tr>
<tr>
<td>15.</td>
<td>77</td>
</tr>
</tbody>
</table>

*Structure of the β-amyloid precursor protein (APP).*
*Structural diagram of Presenilin proteins.*
*Diagram of the hippocampus.*
*Paired helical filament (PHF) structural diagram.*
*Microtubule associated proteins.*
*Tau isoforms.*
*Tau amino acid sequence.*
*Tau phosphorylation sites.*
*Table of tau protein kinases.*
*Tau antibodies.*
*Western blot analysis of fetal and adult rat brain homogenates.*
*Western blot analysis of isolated tau protein.*
*Table of AD and normal cases.*
*Immunohistochemical staining of Alzheimer's disease and normal brain sections.*
*MPM2 staining of neuropathological lesions in AD brain.*
| 16. | Colocalization of MPM2 and AT8 staining in serial sections of AD brain samples | 79 |
| 17. | Immunostaining of AD brain sections with other neuronal MAP antibodies | 81 |
| 18. | PHF purification scheme | 83 |
| 19. | Immunoblot analysis of isolated PHF preparation | 84 |
| 20. | Immunoblot of PHF-tau from separate AD brain samples | 86 |
| 21. | Dephosphorylation of PHF-tau | 88 |
| 22. | MPM2 reactivity of phosphorylated tau | 118 |
| 23. | Immunoblot analysis of *in vitro* phosphorylation of htau40 | 120 |
| 24. | Time course phosphorylation of htau40 in the presence of microcystin | 122 |
| 25. | Time course phosphorylation of htau40 in the absence of microcystin | 124 |
| 26. | Temporal sequence of tau phosphoepitope formation in the presence of microcystin | 126 |
| 27. | Temporal sequence of tau phosphoepitope formation in the absence of microcystin | 128 |
| 28. | *In vitro* phosphorylation of htau40 using fractions from microtubule purification | 130 |
| 29. | Phosphorylation of htau40 using fetal and adult brain kinase extracts | 132 |
| 30. | Recombinant human tau isoforms | 162 |
| 31. | Purification of recombinant human tau isoforms | 163 |
| 32. | Immunoblot analysis of phosphorylated htau24 and htau34 | 165 |
| 33. | MPM2 reactive sequences | 167 |
34. Immunoreactivity of PHF-tau with polyclonal antibodies PMB1 and PTE1.............................................................................................................168
35. Immunoreactivity of in vitro phosphorylated htau40 with PMB1 and PTE1 polyclonal antibodies...........................................................170
36. MPM2 epitope model and potential sites on tau.................................172
37. Tau deletion mutants.............................................................................173
38. Tau deletion mutant purification scheme..............................................174
39. Purification of tau deletion mutants......................................................175
40. Immunoblot analysis of in vitro phosphorylated tau deletion mutants...............................................................................................................177
ABBREVIATIONS

Aβ  β-amyloid
AD  Alzheimer's disease
apo Apolipoprotein
APP β-amyloid precursor protein
BCA Bicinchoninic acid
CaM kinase II Ca^{2+}/calmodulin-dependent kinase
CBS Crude brain supernatant
cdc and cdk Cyclin-dependent kinase
CHO Chinese hamster ovary cells
COOM Coomassie
COS African green monkey kidney cells
C1S Single-cycled microtubule supernatant
C2S Twice-cycled microtubule supernatant
DLB Diffuse Lewy Body disease
DTT Dithiotreitol
E19 Embryonic day 19
ELISA Enzyme linked immunosorbent assay
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial Alzheimer’s disease</td>
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<tr>
<td>FKBP</td>
<td>FK506 binding protein</td>
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<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<td>GSK</td>
<td>Glycogen synthase kinase</td>
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<tr>
<td>HeLa</td>
<td>Human epithelial carcinoma cell line</td>
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<tr>
<td>H1S</td>
<td>Microtubule depleted warm supernatant</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthio-β-galactoside</td>
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<tr>
<td>kDa</td>
<td>Kilodaltons</td>
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<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
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<td>LA</td>
<td>Luria agar</td>
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<td>LAN5</td>
<td>Neuroblastoma cell line</td>
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<tr>
<td>LB</td>
<td>Luria broth</td>
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<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein/extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholino] ethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino] propanesulfonic acid</td>
</tr>
<tr>
<td>MPP</td>
<td>MPM2-reactive phosphoproteins</td>
</tr>
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<td>MT</td>
<td>Microtubule</td>
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<td>MW</td>
<td>Molecular weight</td>
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<td>Abbreviation</td>
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<tr>
<td>NCLK</td>
<td>Neuronal cdc2-like kinase</td>
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<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NIMA</td>
<td>Never in mitosis A kinase</td>
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<tr>
<td>NT</td>
<td>Neuropil threads</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PC12</td>
<td>Rat pheochromocytoma cell line</td>
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<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filaments</td>
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<tr>
<td>PIPES</td>
<td>Piperazine-N, N'-bis[2-ethanesulfonic acid]</td>
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<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<td>PP1</td>
<td>Protein phosphatase 1</td>
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<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
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<tr>
<td>PPIase</td>
<td>Peptidyl-prolyl isomerase</td>
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<td>PS</td>
<td>Presenilin</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SF</td>
<td>Straight filaments</td>
</tr>
<tr>
<td>SHSY-5Y</td>
<td>Neuroblastoma cell line</td>
</tr>
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<td>SP</td>
<td>Senile plaques</td>
</tr>
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<td>3T3</td>
<td>Mouse fibroblast cell line</td>
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</tbody>
</table>
INTRODUCTION

"I remember when......" is a common phrase everyone uses when fondly recalling important moments and people in their lives. However, individuals affected with Alzheimer's disease (AD) rarely remember events that occurred months or days before or even people they have known all their lives. It has been estimated that over 100,000 people in the United States suffer from AD and that as the human life-span increases and the baby-boomer generation ages more people will be afflicted with this devastating disease. Furthermore, approximately 5% of the population over the age of 65 will develop AD and the percentage rises to 10-20% for individuals over the age of 80 (Lendon et al., 1997). AD is the most common form of dementia and the fourth leading cause of death in the United States after heart disease, cancer, and stroke. This disease is characterized by severe intellectual deterioration of an adult beginning with memory loss that progressively worsens over a 6 to 20 year time span. Eventually, these individuals are unable function independently. The commonly observed cognitive changes in individuals afflicted with AD are: 1) impairments in memory, 2) disturbances in language use and perception, 3) the inability to learn new skills, solve problems, think abstractly, and make judgements, and 4) personality changes such as paranoia (Marotta et
al., 1992). The earliest clinical symptom is the inability to retain information after a brief delay. For example, picking up of keys to the car and not knowing what they are used for or how to drive the car. Currently, there is no effective treatment for AD or known cause, although a significant amount of research has identified several biological components contributing to the disease.

**General Pathological and Molecular Hallmarks of AD**

The neuropathological characteristics associated with AD are senile plaques (SP), neurofibrillary tangles (NFT), cerebrovascular amyloidosis, and selective neuronal loss primarily in the temporal cortex and hippocampus (reviewed in Clark and Goate, 1993). The SPs are primarily extracellular deposits consisting of a central core of β-amyloid (Aβ) fibrils 4-8 nm in diameter (discussed in detail below) (Merz et al., 1983). The central core of SPs is surrounded by a rim of dystrophic neurites together with reactive microglia and astrocytes. Over 90% of individuals with AD also have deposits of Aβ within the microvasculature of the brain; thus, exhibiting cerebrovascular amyloidosis (Marotta et al., 1992). The other characteristic AD lesion, NFTs are aggregates of two 10 nm protein filaments twisted around one another in a helical fashion called paired helical filaments (PHF) (Kidd et al., 1964). NFTs are intracellular deposits accumulating within the cytoplasm of the neuronal cell bodies or soma of the hippocampus. In addition, other cellular proteins such as neurofilament proteins, vimentin, actin, ubiquitin, MAP2, MAP1B, tau, and Aβ have also been found associated with NFTs by
immunohistochemical analysis (Hasegawa et al., 1990; Takahashi et al., 1991; Ulloa et al., 1994; Wischik et al., 1995). As the neuron dies these insoluble aggregates are deposited within the extracellular space were they are referred to as ghost tangles or neuropil threads (NT). Furthermore, the dystrophic neurites associated with the SPs contain PHFs and are believed to be regions of degenerating axons. Recently, another neuropathological lesion was identified by immunohistochemical analysis with antibodies raised against extracts from PHF-rich fractions that have been called AMY plaques (Schmidt et al., 1997). These new antibodies did not recognize NFTs and only labeled brains that contained at least a few Aβ-rich plaques with or without associated neurofibrillary pathology. Superficially, the size and morphology of the AMY plaques resembled SPs; however, the AMY plaques did not contain Aβ cores and appear to be composed of a novel 100 kDa protein. How the AMY plaques form and their relationship to the pathogenesis of AD remains to be determined. All of the pathological features of AD, except AMY plaques, occur during the normal aging process but to a significantly lesser degree.

The underlying molecular mechanism for the development of AD is still a mystery; however, experimental research has yielded some potential and interesting hypotheses. The two main areas of research focus on the classical pathological hallmarks of AD, the Aβ containing senile plaques and NFTs. In addition, other proteins and processes such as presenilins, apolipoprotein (apo) E, proteases, and
oxidative stress have been implicated in AD and may contribute to the progression and molecular mechanism of the disease. Several of these proteins and their associated abnormalities were identified by genetic analysis of families with a high incidence of AD, commonly referred to as familial AD (FAD). FAD can be divided into two groups based on the age of onset of the disease, with the late-onset form occurring at a mean age of 65 (Lendon et al., 1997) whereas early-onset FAD can occur as early as 30 years (Schellenberg, 1995). Many of these families have mutations in the amyloid precursor protein (APP) or presenilin proteins PS1 and PS2, and carry the apoE4 risk factor allele. Sporadic AD, on the other hand, is the most common form of the disease affecting individuals >65 years of age (Schellenberg, 1995). Having a wide variety of potential causes with no clear genetic abnormalities present in the overall population, a direct cause for development of sporadic AD has not been identified. Thus, these differences between FAD and sporadic AD have led to opposing hypotheses and uncertainty regarding the actual mechanism(s) involved in the etiology of the disease.

**Apolipoprotein E (apoE) and AD**

A risk factor for the development of AD is the inheritance of a specific allele of the apoE protein, apoE4, in both FAD and sporadic AD cases (reviewed in Schellenberg, 1995; Strittmatter and Roses, 1995). ApoE is a 34 kDa plasma lipoprotein which plays a role in the transport, uptake, and redistribution of cholesterol (Masliah et al., 1996). In the brain, which is the second major site of
apoE synthesis, apoE is believed to be involved in maintaining synaptic integrity during aging and after injury. ApoE is primarily synthesized and secreted by the glia cells (Masliah et al., 1996). The secreted apoE is taken up by the neurons and concentrated at the synaptic terminals and neuromuscular junctions where it is believed to play a role in plasticity. It has been suggested that apoE has multiple functions including: 1) stabilization of the neuronal cytoskeleton, 2) a role in transport of esterified cholesterol to damaged neurons undergoing regeneration and formation of new synapses, 3) regulation of interactions between neurons and the extracellular matrix, and 4) regulation of intracellular calcium levels (Masliah et al., 1996). These functions of apoE are thought to be modulated by the interaction of apoE with various receptors such as the low density lipoprotein receptor. It is also important to point out the behavioral studies done with mice deficient in apoE (Gorden et al., 1995). These animals have significant learning deficits as a result of age-dependent neurodegeneration of the synaptodendritic complexes.

The involvement of apoE in AD is demonstrated through localization of apoE to both senile plaques and NFTs (Namba et al., 1991; Wisniewski and Frangione, 1992; Wisniewski et al., 1993; Han et al., 1994). There are three common alleles of the apoE protein, apoE2, apoE3, and apoE4. The differences between the three alleles consists of single base changes in the codons encoding amino acids 112 and 158. The apoE2 allele contains two Cys residues at both of the sites whereas these amino acids are changed to Arg in the apoE4 isoform (Strittmatter et al.,
1993a). The apoE3 isoform contains a Cys and an Arg at amino acids 112 and 158, respectively, and is the most common allele found in the population, 78% (Strittmatter and Roses, 1995). The apoE2 and E4 alleles are less common making up 7% and 15% of the population, respectively. The association of the apoE4 allele with AD was first reported in late-onset FAD (Strittmatter et al., 1993a). Additional research revealed a dose-dependent increase in the risk for AD through the decrease in the age of onset (Corder et al., 1993) and rapid progression of the disease. Thus, individuals who inherit two copies of the apoE4 allele have a higher risk of developing AD than individuals with one copy of the E4 allele or individuals with two copies of the E3 allele. On the other hand, individuals who inherit the apoE2 allele have a lower frequency of developing AD than individuals with the E3/E3 genotype (Corder et al., 1994). Therefore, the inheritance of the apoE2 allele appears to confer a protective effect against development of AD.

As stated above, apoE associates with the classical neuropathological lesions of AD and is a risk factor increasing by as much as 50% the chance for development of AD (Lendon et al., 1997). Furthermore, approximately 64% of AD cases have the apoE4 genotype suggesting that an abnormal function of the apoE protein may be contributing to the development and progression of AD. The actual mechanism of how apoE genotypes lead to AD is still unclear; however, several studies suggest the involvement of specific isoforms in the abnormal neuronal regulation of Aβ clearance, phosphorylation of cytoskeletal proteins, intracellular
calcium levels, synapse remodeling, and altered cholesterol in neuronal membranes (reviewed in Masliah et al., 1996). Interestingly, apoE can bind to both Aβ and tau which are components of the senile plaques and NFTs, respectively. In vitro studies indicate that the various apoE isoforms have different binding affinities for Aβ and differential affects on Aβ deposition (Strittmatter et al., 1993b; Lendon et al., 1997). The inheritance of the apoE4/E4 allele is associated with an increase in the deposition of Aβ into senile plaques compared to the E3/E3 genotype (Kamboh, 1995). One study shows that apoE4 binds more rapidly and stronger to Aβ than the apoE3 isoform; however, another report describes the opposite scenario (Schellenberg, 1995). Thus, the interaction between apoE isoforms and Aβ is uncertain and whether this interaction is relevant to pathogenesis of AD or occurs in vivo remains to be determined. Another difference between apoE isoforms is seen with the association of ApoE and the cytoskeletal protein tau. In vitro studies show that apoE3 binds with high avidity to tau and MAP2c, another cytoskeletal protein (Strittmatter et al., 1994; Kamboh, 1995; Masliah et al., 1996). However, the apoE4 isoform does not bind to either of these proteins. It has been suggested that apoE3 and possibly apoE2 act to prevent formation of NFTs by binding to tau and protecting it from becoming hyperphosphorylated (formation of NFTs and involvement of tau are discussed in detail later). Lastly, apoE isoforms appear to have opposing effects on neurite outgrowth with apoE3 stimulating extension and
apoE4 repressing outgrowth (Masliah et al., 1996). The inheritance of the apoE4 allele indicates that a defective apoE protein may have some influence on neurodegeneration associated with AD.

β-amyloid (Aβ)-rich senile plaques

As stated earlier, the SPs are composed of Aβ fibrils which are aggregates of 4 kDa peptides of 42 or 43 amino acids referred to as Aβ42/43. A smaller Aβ peptide, Aβ40, is the soluble form expressed normally by cells, and this form is not prone to aggregate into fibrils. The Aβ peptides are created from proteolytic digestion of a larger transmembrane protein called the β-amyloid precursor protein (APP) (Figure 1). APP is encoded by a single gene located on chromosome 21 which undergoes alternative splicing giving rise to at least 5 different isoforms ranging in size from 563-770 amino acids (reviewed by Clark and Goate, 1993). The APP770 protein is the most abundant form; however, the APP695 isoform is predominantly expressed in the central nervous system. APP was linked to AD through observations that adults over the age of 30 with Down’s syndrome, who carry three copies of chromosome 21, develop AD. These observations led to genetic linkage studies and cloning of the APP gene in FAD. Additional genetic analysis of various families with AD revealed missense mutations occurring within the APP gene suggesting a pathological role for APP in the development of AD.
APP is a cell surface protein with a large N-terminal extracellular domain, a single transmembrane domain, and a short C-terminal cytoplasmic tail (Figure 1). The Aβ sequence is a small fragment of APP starting at amino acid 672 (numbering according to the longest isoform) within the extracellular domain and terminating 40-42 amino acids downstream. The last 11-15 amino acids of the Aβ peptides are derived from the amino acid sequence located within the transmembrane region of APP. The proteolytic processing of APP follows two different pathways yielding either soluble APP and smaller fragments which are incapable of aggregating into SPs or Aβ peptides (Figure 1) (reviewed by Haass and Selkoe, 1993). The normal secretory processing of APP involves cleavage of this molecule at amino acid 16 within the Aβ sequence. This cleavage is performed by the enzyme α-secretase which releases soluble APP and retains a small 10 kDa fragment corresponding to the C-terminus within the plasma membrane (Haass and Selkoe, 1993). The 10 kDa fragment can be further cleaved by another enzyme γ-secretase into two additional fragments of 3 and 7 kDa. The α-secretase cleavage of APP appears to occur in a sequence independent manner located at the cell surface or intracellularly in endosomes or lysosomes. Thus, the cleavage of APP by α-secretase prevents the formation of Aβ peptides and subsequent deposition into SPs. The soluble APP is reported to have numerous biological functions including: 1) regulating intracellular Ca^{2+} levels and neurite outgrowth, 2) modulating glutamate
responses, 3) protecting neurons against excitotoxic or ischemic insults, 4) acting as a trophic factor for non-neuronal cell proliferation, and 5) regulation of proteases, blood coagulation, and cytokine release (Mattson et al., 1993).

An alternative cleavage pathway creates a truncated version of soluble APP due to the proteolytic activity of another enzyme called β-secretase. The β-secretase cleavage of APP is highly sequence-dependent severing the peptide bond linking amino acids 671 and 672. This precise cut of the full-length APP molecule exposes the N-terminus of the Aβ peptides. Subsequent proteolysis by γ-secretase creates Aβ peptides ranging in length from 39-43 amino acids and a residual fragment consisting of the extreme C-terminus of APP (Haass and Selkoe, 1993). The production of the longer Aβ fragments, Aβ42/43, results in aggregation, Aβ fibril formation, and deposition into SPs. The release and aggregation of Aβ42/43 appears to have several effects on the surrounding cells including neurotoxicity, alterations in neurite outgrowth, promotion of the release of cytokines, and destabilization of neuronal Ca^{2+} homeostasis possibly through the formation of Ca^{2+} conducting pores in the plasma membrane (Mattson et al., 1993). Thus, a shift in the normal processing of APP that increases the levels of Aβ42/43 and decreases the levels of soluble APP could lead to a cellular environment that stimulates or enhances neurodegeneration.
All of the AD associated mutations within APP appear to decrease the age of onset of AD. It is estimated that 5-20% of the early-onset FAD cases are caused by these mutations (Lendon et al., 1997). The mutations can be grouped into four sets based on amino acid substitutions at 670/671, 693, 716, and 717 (Figure 1). The double mutant at amino acids 670/671 is located at the β-secretase cleavage site and is suggested to increase the affinity of APP for cleavage by this enzyme. Furthermore, the 670/671 mutation enhances the secretion of both Aβ40 and Aβ42/43 by approximately 3-fold. Mutations at amino acid 717 or 716 only show a modest increase, 1.5-fold, in the production of Aβ42/43 with no change in Aβ40 levels. However, the three different amino acid substitutions occurring at position 717 of APP may influence γ-secretase activity thereby generating the longer Aβ42/43 forms which aggregate more readily into fibrils. Transgenic mice overexpressing the human APP double mutant and the V717F substitution have increased levels of Aβ42/43 and spontaneously deposit Aβ in the brain with increased age. These animals also show impairments in memory; however, they do not show signs of NFT formation or neuronal loss (Lendon et al., 1997). Thus, the mutations found within the APP gene in FAD, and the increased dosage of this gene that is present in Down’s syndrome, have led researchers to propose the Aβ hypothesis for the development of AD. This hypothesis states that the increase of Aβ deposition due to alterations in the normal proteolytic processing and increased dosage of APP are responsible for neurodegeneration associated with AD through toxicity of Aβ42/43.
Presenilin proteins PS1 and PS2

Another player involved in the development of AD was discovered through genetic analysis of early-onset FAD cases that did not show linkage to the APP gene on chromosome 21, but rather to chromosome 14 (reviewed by Haass, 1997). Molecular cloning of this region yielded the presenilin protein (PS)-1 and further genetic analysis found a second protein PS-2 located on chromosome 1. Both PS proteins contain point mutations, with more than 35 different mutations identified in PS-1 and only 2 mutations contained within PS-2. All of the mutations found in the PS proteins are associated with FAD and occur at positions conserved between the two molecules, thus implying that these mutations effect the proper functioning of the proteins. Furthermore, the mutations within the PS-1 protein appear to be responsible for the most aggressive form of FAD with clinical signs and AD pathology evident as early as 35 years of age (reviewed by Haass, 1997).

The PS proteins are integral membrane proteins that are structurally similar, having approximately 63% homology across the entire protein and about 95% homology within their transmembrane domains. The overall structure of PS is comprised of 6-8 transmembrane domains interconnected by hydrophilic loops (Figure 2). The N-terminal and C-terminal domains, and a large loop region between transmembrane domains 6 and 7, are orientated towards the cytoplasm or nucleoplasm of the cell. The two proteins differ from one another in the N-terminal and large loop domains were they have the least homology. The PS-1 loop
domain is larger than the corresponding region of PS-2. Antibodies raised against the PS proteins have localized them to the endoplasmic reticulum and Golgi (Haass, 1997). However, there is some controversy over the PS cellular distribution, with one recent report showing PS localization to the cell surface (Dewji and Singer, 1997) and another report localizing PS to the nuclear membrane, interphase kinetochores, and centrosomes (Li et al., 1997). The controversy over cellular location has led to speculation for numerous functions of the PS proteins.

The most documented and convincing normal biological function of PS stems from the observation that human PS-1 shows striking structural homology to the \textit{C. elegans} sel-12 gene product indicating a possible conserved evolutionary function. The sel-12 protein is involved in the Notch signaling pathway, and \textit{C. elegans} lacking this gene can be rescued by either human PS. These results suggests that the PS proteins are involved in the Notch signaling pathway, which is required for normal somite segmentation and subsequent development of the axial skeleton. Further evidence for PS involvement in this pathway was shown with PS-1 knock-out mice which die shortly after birth. These animals exhibit defects in skeletal development, such as impaired rib cage formation, as well as impaired neurogenesis and neuronal cell death.

The majority of PS mutations associated with FAD are located within the second transmembrane domain and the large hydrophilic loop domain. It has been
suggested that the mutant PS proteins alter the proteolytic processing of APP. Analysis of FAD-associated PS mutations in cells transfected with mutant cDNAs reveals a significant increase in the secretion of Aβ42 levels over Aβ40 (Haass, 1997). Similar results were also obtained from experiments using transgenic mice overexpressing PS-1 mutant proteins. Furthermore, it has been speculated that PS might bind to the immature form of APP that is localized to the endoplasmic reticulum where mutations within the PS proteins could alter this binding and the normal APP processing resulting in the production of Aβ peptides. This mechanism is supported by the finding that abundant levels of Aβ42 are generated in neurons, and the site of Aβ synthesis is the endoplasmic reticulum (Tienari et al., 1996). Therefore, the Aβ hypothesis can be expanded to the following simplified scenario: 1) PS missense mutations increase the production of Aβ42 and total Aβ peptides, 2) the Aβ42 peptides aggregate into fibrils which eventually form SPs, 3) the SPs are toxic to the surrounding neurons and elicit an inflammatory response which causes the release of cytokines, 4) toxicity and/or cytokines could cause alterations in neuronal Ca^{2+} homeostasis, which in turn could affect intracellular regulatory pathways involving kinases and phosphatases, and 5) eventually lead to hyperphosphorylation of tau, formation of NFTs, and neurodegeneration (see below).

Alternatively, it was suggested that PS have a dual function in neurons involving both the cytoplasmic and nuclear membranes (Li et al., 1997).
Colocalization of PS with interphase kinetochores and centrosomes indicated that these proteins may have functions in mitosis and in chromosome organization and segregation. Li et al. (1997) stated that PS may act as kinetochore binding proteins or receptors that anchor the chromosomes to the nuclear membrane during interphase. Furthermore, they proposed that mutations within the PS genes that are associated with FAD could affect the ability of these proteins to properly link the chromosomes to the nuclear membrane. They further speculated that the progressive accumulation of chromosome 21 during an individuals life could be responsible for the development of both genetic and sporadic forms of AD. The authors supported this speculation with two additional observations: 1) trisomy 21 Down's syndrome patients develop AD neuropathology by age 30-40, and 2) fibroblast cells from AD patients carrying FAD mutations in either PS protein exhibit an increased frequency of trisomy 21. Whether this hypothesis is true and the importance and function of PS in chromatin organization remains to be determined.

**Pathology of Neurofibrillary degeneration**

An alternative to the Aβ hypothesis deals with the formation of NFTs and subsequent neuronal degeneration. The NFTs found within pyramidal neurons of the hippocampus correlate with the extent and severity of AD and indicate a disruption in neuronal connections in and out of the hippocampus. Thus, it is not surprising that individuals clinically diagnosed with AD exhibit loss of cognitive functions and memory, since the hippocampus is the region of the brain involved in
memory formation. A comparison of individuals with varying degrees of AD show a progressive accumulation of NFTs throughout the hippocampal formation that follows a predictable non-random sequence (Braak and Braak, 1995; Wischik et al, 1995). The initial region affected is the transentorhinal cortex which shows mild neurofibrillary changes and slow development of NFTs and NTs with patients exhibiting no clinical symptoms (Figure 3). As the disease progresses, there is an accumulation of NFTs in a sequential-like fashion from the transentorhinal and entorhinal cortices towards the CA1 region of the hippocampus. At this stage of the disease, individuals exhibit clinical impairment of cognitive functions and personality changes. The most severe stages of AD show further spreading of the NFTs to the CA2, CA3, and CA4 regions, the subiculum, and isocortical areas as well as severe destruction of the neocortical association regions. Interestingly, the granule cells of the dentate gyrus and the pyramidal cells in the CA3 region of the hippocampus are resistant to degeneration (DeLacoste and White, 1993; Morrison and Hof, 1997) with the CA3 pyramidal neurons only exhibiting occasional tangle formation (DeLacoste and White, 1993).

What is intriguing about AD is the specific destruction of neurons in layers of the hippocampus that are involved in cortico-cortical connections. The pyramidal neurons within the hippocampus are interconnected by extension of long projections forming a circuit or input-output pathway (Figure 3). Three distinct pathways referred to as the perforant, mossy fiber, and Schaffer collateral paths make up the
major portion of this network or the trisynaptic loop (Kennedy and Marder, 1992; DeLacoste and White, 1993; Wischik et al., 1995). The hippocampus receives input from the association cortex via the pyramidal cells in layer II of the entorhinal cortex. These primary receiving neurons of the perforant pathway project their axons mainly to the dendrites of the granule cells of the dentate gyrus in addition to dendrites of pyramidal neurons within the CA3, CA2, CA1, and subiculum regions of the hippocampus. The granule cell axons or mossy fibers synapse on the dendrites of the CA3 pyramidal neurons, which extend branched axons with one branch leaving the hippocampus and the other branch, the Schaffer collateral path, projecting to the CA1 pyramidal neurons. Projections from the CA1 pyramidal neurons synapse on dendrites of the subiculum neurons, which extend their axons to pyramidal neurons within layer IV of the entorhinal cortex or the output neurons. The hippocampal circuit is completed with the axons of the output neurons projecting to other regions of the brain. The progression of AD or destruction of pyramidal neurons seems to follow along these connections eventually disrupting both the input and output circuitry and isolating the hippocampus from the association cortices. Two hypotheses address the manner in which these connections are degraded (DeLacoste and White, 1993). First, the retrograde hypothesis states that the initial damage occurs at the axon terminals in the entorhinal cortex and spreads towards the cell body. This initial damage may be the result of the lack of trophic factors or exposure of the neurons to some toxic insult such as Aβ deposits and/or oxidative stress. The alternative hypothesis, orthograde, implies the opposite
scenario were the initial damage occurs within the cell bodies of the entorhinal cortex pyramidal neurons followed by progression along the axons towards the synapse. This hypothesis seems to correspond more directly with the distribution and accumulation of NFTs throughout the hippocampus in AD, however, the actual path of degeneration may be a combination of both processes.

**NFTs and microtubule-associated proteins**

Although the accumulation of NFTs seems to progress in a sequential fashion, the molecular or cellular pathways involved in formation of NFTs are more complicated and are not fully understood. What is known about the formation of NFTs implies yet another hypothesis for the underlying mechanism of AD, the tau hypothesis. PHFs, the primary structural component of NFTs, consist of a protease-resistant core surrounded by a fuzzy coat which can be removed by pronase digestion (Wischik et al., 1988; Wischik et al., 1995). Both the PHF fuzzy coat and core are composed of the microtubule-associated protein (MAP) tau (Grundke-Iqbal et al., 1986a; Wischik et al., 1988). The fuzzy coat consist mainly of the N-terminal half and a small segment of the extreme C-terminus of tau (Figure 4). The protease-resistant core is composed of self-assembled 12 kDa peptides derived from the microtubule (MT) binding repeats of tau (discussed below) (Jakes et al., 1991; Wischik et al., 1995). The 12 kDa tau peptides originated from both three and four repeat isoforms, and are restricted in length to three MT binding repeats that
are 14-16 amino acid residues out of phase with each other depending on which tau isoform, 3 or 4 repeats, the peptide was derived from (Wischik et al., 1995).

Tau is only one member of a larger group of microtubule-associated proteins including three other neuronal proteins, MAP1A, MAP1B, and MAP2, and the somatic protein MAP4 (Figure 5). These proteins directly interact with and regulate microtubule (MT) assembly/disassembly. MTs are highly dynamic polar filaments that make up one of the three cytoskeletal components in all eukaryotic cells. MTs are involved in spindle formation and chromosome segregation during mitosis, cell shape determination and movement, intracellular transport of organelles along axons and dendrites, and play roles in growth and development of neurons (Brinkley, 1997; Vega and Solomon, 1997). These highly dynamic structures assemble from cytoplasmic pools of α- and β-tubulin. Briefly, heterodimers of α- and β-tubulin assemble in a head-to-tail fashion to form a protofilament that associates with 12 other protofilaments arranged to form a cylindrical MT. Axonal MTs are extremely long and stable with lengths > 100 μm (Hammerschlag et al., 1994). MAPs promote the assembly of MTs and stabilize them against depolymerization by binding along the length of the MT. The effects of MAPs on the stiffness or rigidity of MTs is dominated by the high molecular weight (MW) MAPs such as MAP1A, MAP1B, MAP2, and MAP4 (Felgner et al., 1997). MAPs can modulate MT rigidity by two possible mechanisms: 1) MAPs bind along the exterior of the MTs in such a way that interlinks two or more tubulin subunits and limits
interdimer movements, or 2) MAPs binding to MTs changes the conformation of the tubulin dimer resulting in stiffening of the polymer due to alterations in interdimer binding (Felgner et al., 1997). The cell biological consequences of MAP-induced stiffening of MTs is essential in neurons were long cellular processes require some type of mechanical stability. The stiffened MTs may contribute to this morphological stability and the structural framework of the axon (Felgner et al. 1997), along with other factors such as the neurofilaments, another cytoskeletal component in neurons. Furthermore, MTs are the principal structure along which fast axonal transport occurs; therefore, loss of stable MTs could lead to impaired signaling processes within the neuron and abnormal synaptic transmission.

The basic structure of the MAPs is divided into the MT binding domain and the projection domain (Figure 5). The MT binding domain is located either in the C-terminal portion of the protein as in MAP2, MAP4, and tau or in the N-terminal region as in MAP1A and MAP1B. The MT binding or assembly domain of MAP2, MAP4, and tau contains 3-4 imperfect repeats of approximately 31 amino acids (Figure 7) with the number of repeats varying due to alternative splicing (Figure 6) (Drubin et al., 1984; Neve et al., 1986; Lee et al., 1988; Lewis et al., 1988; Himmler et al., 1989; Goedert et al., 1989a; 1989b; Aizawa et al., 1990; West et al., 1991; Capin and Bulinski, 1991; Doll et al., 1993). These repeats can be subdivided into an 18 amino acid conserved motif (Figure 7, bold amino acids) and a 13-14 amino acid spacer section that is less highly conserved. MAP1A and MAP1B have a
different MT binding domain containing 11 or 21 repeats, respectively, of the amino acid motif Lys/Arg-Lys/Arg-Glu/Asp (Noble et al., 1989; Langkopf et al., 1992). Also, MAP light chain proteins associate with the MT binding repeats of MAP1A and MAP1B, and are speculated to aid in the MT-MAP interaction. The projection domain makes up the rest of the protein and extends out into the cytoplasm away from the MT. In the case of tau, the smallest MAP, the projection domain protrudes from the MT surface approximately 19 nm (Hirokawa et al., 1988). Also, MAP2, MAP4, and tau contain a proline-rich hinge domain located between the projection and MT binding domains. Furthermore, MAPs are phosphoproteins with many of their functions being regulated by phosphorylation. For example, the phosphorylation of MAPs interferes with the ability of MAPs to stabilize MTs by decreasing their affinity for the MTs (Brugg and Matus, 1991; Shiina et al., 1992; Drechsel et al., 1992; Biernat et al., 1993; Brandt et al., 1994; Ookata et al., 1995; Trinczek et al., 1995). In addition to stabilizing MTs, MAPs may function to sequester or anchor other cytoplasmic proteins, such as kinases and phosphatases to certain regions of the cell (Obar et al., 1989; Sontag et al., 1995; Ookata et al., 1995). For example, cdc2 kinase is localized to MTs through the binding of cyclin B to the proline-rich region of MAP4 (Ookata et al., 1995). In addition, tau has recently been shown to co-elute with neuronal cdc2-like kinase from a bovine brain extract (Mawal et al., 1997) and act as a MT targeting subunit for protein phosphatase 1 (PP1) (Liao et al., 1997). Thus, the projection domain of MAPs may be indirectly involved in regulating signal transduction cascades by binding,
releasing, or modulating sequestered signaling enzymes. It has also been suggested that MAPs may function as physical spacers between MTs and modulate their surface charge (Preuss et al., 1997). The transfection or microinjection of MAPs into cells produces bundling of endogenous MTs implying that MAPs are directly responsible for cross-linking between adjacent microtubules through interaction of their projection domains (Lewis et al., 1989; Kanai et al., 1992). However, additional mechanisms may also be responsible for MT bundling within the cell.

Another important function of MAPs are their roles in differentiation and neurite outgrowth, which also appears to be regulated by their state or level of phosphorylation. Tau and MAP2 are believed to be involved in the polarization of axons and dendrites during early development (Matus et al., 1981; Binder et al., 1985; Riederer and Matus, 1985; Peng et al., 1986; Papasozomenos and Binder, 1987; Brion et al., 1988; Migheli et al., 1988; Trojanowski et al., 1989). Suppression of MAP2 results in inhibition of minor neurite formation (Cáceres et al. 1992) whereas suppression of tau results in inhibition of axon-like processes (Cáceres and Kosik, 1990) in neuronal cultures. Tau appears to be localized throughout the neuronal cytoplasm; however, it is enriched in the growth cones and minor processes (Deitch and Banker, 1993; Black et al., 1996). During differentiation of PC12 cells and rat hippocampal neurons, tau was present not only associated with MTs, but also at the periphery of the growth cone (Brandt et al., 1995). Further, tau
localization to the distal axon is an early event in the development of polarity becoming present in cultured rat hippocampal neurons by day 2 (Kempt et al., 1996). In comparison, MAP2 is excluded from the axon much later, after day 8, suggesting different mechanisms by which tau and MAP2 are compartmentalized and different functions (Kempt et al., 1996). Interestingly, the phosphorylation state of tau regulates its localization during neurite outgrowth. There appears to be a proximal-distal gradient of tau phosphorylation along the axon with tau being highly phosphorylated in the somatodendritic compartment and proximal axon. Towards the synapse, the phosphorylation state of tau decreases with tau becoming dephosphorylated at the distal axon and growth cone (Mandell and Banker, 1996). Thus, modulation of the phosphorylation state of tau, possibly through mitogenic signaling events, may be responsible for the establishment of polarity in neurons.

In addition to interactions with MTs, MAPs may have additional non-microtubular functions. In particular, tau has been found in the nucleus associated with the nucleoli (Loomis et al., 1990; Wang et al., 1993; Preuss et al. 1997). It has been suggested that nuclear tau, which makes up 16% of the total tau protein in LAN5 cells, is involved in the transport of newly synthesized ribosomes from the nucleus into the cytosol (Greenwood and Johnson, 1995). This hypothesis is supported by the finding that tau interacts with ribosomes (Rendon et al., 1990). Tau is also associated with the mitochondria (Rendon et al., 1990), and both the ER (Lu and Wood, 1993a) and plasma (Brandt et al., 1995) membranes. The region
of tau that interacts with the neural plasma membrane is the N-terminus or projection domain suggesting a role for this MAP in mediating MT-plasma membrane interactions via association with an intracellular membrane-bound protein (Brandt et al., 1995). Thus, the functions of MAPs appear to be diverse and not restricted to interactions with MTs.

**Tau and AD**

As stated above, the neuronal and smallest MAP, tau is involved in the progression of AD through its self-assembly into PHFs. Tau is comprised of a heterogeneous family of six isoforms that are expressed in the adult brain ranging in size from 55-65 kDa (Figure 6) (Goedert et al., 1989a; 1989b; Kosik et al., 1989). Only one of these isoforms, the smallest or S3, is expressed during fetal development. The remaining isoforms or adult isoforms are expressed starting around postnatal day 10 and persist into adulthood (Brion et al., 1993; Smith et al., 1995). All six of the tau isoforms are encoded by a single gene with the largest isoform containing 441 amino acids (Drubin et al., 1984; Neve et al., 1989). The six isoforms arise from alternative mRNA splicing and differ by having either 3 or 4 MT binding repeats in the C-terminus of the protein and 0, 1, or 2 N-terminal inserts in the projection domain (Goedert et al., 1989a; Himmler, 1989). The basic structure of tau can be divided into regions (numbering according to the longest isoform): 1) the assembly domain containing the MT binding repeats range from amino acids 198-441, 2) the projection domain ranges from the N-terminus to amino acid 197,
3) a proline-rich and basic region spanning amino acids 151-244, and 4) targeting domains that flank the MT binding repeats, which include the proline-rich region and amino acids 369-400 (Gustke et al., 1994; Preuss et al., 1997). The two domains involved in the interaction of tau with the MT surface are the targeting domains and the MT binding repeats, as proposed by the jaws model (Gustke et al., 1994; Preuss et al., 1997).

The jaws model of MAP binding states that the targeting domains are responsible for correct positioning of tau and other MAPs along the MT surface and tight binding; however, the MT binding repeats are necessary for promoting microtubule assembly through nucleating and stabilizing the MTs. Both of these domains work in conjunction and are essential for a functional tau-MT interaction. Further support for the jaws model has been presented in a recent study using deletion mutants of MAP1A. The MAP1A construct lacking the MT binding repeats bound tightly to the MTs indicating that the flanking regions are responsible for binding of MAP1A to the MT surface (Vaillant and Brown, 1997). Interestingly, the tau-MT interaction can be modulated by phosphorylation of Ser/Thr residues within the targeting domains and select amino acids in the MT binding repeats (discussed below).

The phosphorylation state of MAPs can regulate their functions, especially their ability to bind and stabilize MTs. Abnormal phosphorylation of tau is believed
to be involved in the formation of NFTs in AD. The tau present in AD or PHF-tau is hyperphosphorylated on 19-21 Ser/Thr sites out of a total of 25 sites (Figures 8 and 9), with all of the sites except three located in the regions flanking the MT binding repeats (Imahori and Uchida, 1997; Lovestone and Reynolds, 1997). Since these regions are responsible for correct placement of tau along the MT surface, phosphorylation of numerous residues within the targeting regions could inhibit MT-tau interactions. For example, phosphorylation of the Ser 396 residue (Figure 8) reduces the affinity of tau to bind to MTs (Bramblett et al., 1993). Furthermore, phosphorylation of the Ser 262 site located within the first MT binding repeat can completely eliminate the association of tau with the MTs (Biernat et al., 1993). Phosphorylation of both Ser 262 and Ser 396 occurs on PHF-tau in addition to many other residues within the flanking regions (Figure 9). PHF-tau is not uniformly phosphorylated at all the possible sites, but is heterogeneously phosphorylated. The extent of phosphorylation varies between individual tau molecules. In this context, the major phosphorylation sites present on PHF-tau are Ser 198, Ser 199, Ser 202, Thr 231, Ser 235, Ser 396, Ser 404, Ser 409, Ser 413, and Ser 422 with the other sites appearing to be partially phosphorylated (Figure 9) (Morishima-Kawashima et al., 1995). Comparing the level of phosphorylation, PHF-tau is phosphorylated to the highest degree followed by fetal and then adult tau. Interestingly, fetal tau is phosphorylated at a total of 13 sites with 12 of these sites also phosphorylated in PHF-tau (Figure 9) (Morishima-Kawashima et al., 1995). On the other hand, normal adult tau is minimally phosphorylated with most of the PHF-
tau sites being phosphorylated to a lesser extent (Garver et al., 1994; Matsuo et al., 1994). Thus, the hyperphosphorylation of PHF-tau is hypothesized to be responsible for the neuronal degeneration seen in AD. A portion of the tau hypothesis (Lu and Wood, 1993b) suggests that PHF-tau causes a defective MT network within neurons through its inability to bind to MTs and stimulate their assembly. This results in the destabilization of the previously stable MT network. Loss of stable MTs would lead to a decrease or inhibition of fast axonal transport, and overtime the neuron would not be able to maintain its distal axonal processes. Thus, a significant reduction in MTs would eventually lead to axonal degeneration.

The mechanism by which hyperphosphorylated tau forms PHFs and subsequently NFTs is still unknown, however, in vitro experiments have implied some intriguing possibilities. The general hypothesis states that hyperphosphorylated tau disassociates from MTs and self-assembles through the interaction of their MT binding repeats forming the insoluble PHF core. These MT binding repeats are arranged in an anti-parallel orientation and may involve strong ionic interactions such as salt bridges (Crowther and Wischik, 1985; Ksiezak-Reding and Yen, 1991). The PHFs further aggregate along with other cellular proteins to form intracellular NFTs within the cytoplasm of pyramidal neurons. Subsequent degeneration of the neurons leads to deposition of the NFTs in the extracellular space. It has been suggested that the preservation of the MT binding repeats is critical for stable PHFs and that self-assembled full-length PHF-tau undergoes
digestion into smaller fragments of approximately 14-17 kDa corresponding to the MT repeats (Figure 4) (Ksiezak-Reding and Yen, 1991). In support of this assumption, extracellular tangles present in AD brains react only with antibodies specific to the MT binding repeats and not N- or C-terminal antibodies (Bondareff et al., 1990). However, straight filaments (SF) also co-exist with PHFs within the NFTs. Some fibrils exhibiting transitional forms of paired helical morphology continuous with stretches of SFs have been observed (Wischik et al., 1985; Perry et al., 1987; Papasozomenos, 1989; Crowther, 1991).

*In vitro* assembly of tau constructs containing only the MT binding repeats can form PHFs independent of phosphorylation, whereas full-length tau does not form PHFs under the same conditions (Wille et al., 1992). This suggests that abnormal phosphorylation of tau induces a conformational change in the molecule that promotes the formation of PHFs, and that the regions flanking the MT binding repeats normally prevent aggregation of tau into PHFs (Wille et al., 1992). Thus, the formation of the PHF core may be dependent on a mechanism in addition to phosphorylation. One study suggests that intermolecular disulfide bridges form at Cys 322, within the third MT binding repeat under oxidative conditions similar to those found in aging neurons (Schweers et al., 1995). This disulfide linkage allows an initial dimerization of individual tau molecules and subsequent PHF assembly (Schweers et al., 1995). Additional *in vitro* data support the requirement for this initial dimerization reaction. It is speculated that the N- and C-terminal tails of tau,
which are acidic, normally fold back onto the positively charged MT binding repeats and protect tau from dimerization (Kampers et al., 1996). Ionic interaction with a polyanion, such as RNA, is thought to change the conformation of tau forming an open or linear state that would facilitate intermolecular dimerization and subsequent assembly into PHFs. In addition, tau isoforms having four MT binding repeats contain two Cys residues that can form an intramolecular disulfide bridge and prevent tau dimerization. Therefore, polyanions such as heparin, polyglutamate, and tRNA can overcome the inhibition on dimerization caused by both the regions flanking the MT binding repeats and the intramolecular disulfide linkages between Cys 322 and Cys 291 occurring in the four repeat tau isoforms (Kampers et al., 1996). Recently, it was reported that sulfated glycosaminoglycans and RNA could stimulate tau phosphorylation by proline-directed kinases NCLK, MAPK, and GSK3β (Hasegawa et al., 1997). Furthermore, NCLK phosphorylation of tau promotes dimerization and formation of intermolecular disulfide linkages (Paudel, 1997b). It is possible that polyanions work synergistically to promote the initial steps in PHF formation by altering the conformation and inducing the phosphorylation of tau. The contribution of individual tau phosphorylation sites towards modulating this dimerization remains to be determined.

Once the PHF core has formed, it has been speculated that PHF-tau acts as a nucleation center that competes with MTs for binding of normal tau (Alonso et al., 1996). The actual "seeds" of the PHF core are soluble hyperphosphorylated
tau/normal tau aggregates, which appear to undergo further phosphorylation and glycosylation that cross-links the tau proteins into PHFs (Alonso et al., 1996; Wang et al., 1996). The binding affinity of PHF-tau for normal tau appears to be stronger than the binding affinity between normal tau and MTs. Moreover, the dephosphorylation of PHF-tau inhibits the interaction with normal tau and the formation of NFTs. Interestingly, soluble hyperphosphorylated tau also sequesters MAP1 and MAP2; however, this association does not lead to the formation of NFTs or long filaments (Alonso et al., 1997). Recent results also indicate that the MT binding repeats of MAP2 can form AD-like filaments in vitro, with these filaments appearing to interact with the ends of PHFs (De Ture et al., 1997). However, it is unclear whether MAP2 filaments have any pathological significance, since they have never been shown to occur in vivo. Thus, the sequestering of MAPs from the MTs into the cytoplasm could lead to a breakdown of the MT network, inhibition of axonal transport, loss of synapses, and retrograde degeneration.

An imbalance in the system regulating the phosphorylation/dephosphorylation of tau in AD brains is probably responsible for the abnormal phosphorylation of PHF-tau and formation of NFTs. The majority of the phosphorylation sites on tau are Ser/Thr-Pro sites implicating the involvement of proline-directed kinases (Figure 8 and 9). Several of these kinases can phosphorylate tau in vitro at sites found on PHF-tau including several members of the mitogen-activated protein kinase (MAPK) family, cyclin-dependent kinases cdk5
and cdc2, and two glycogen synthase kinases GSK-3α and GSK-3β (Drewes et al., 1992; Goedert et al., 1992a; Ledesma et al., 1992; Hanger et al., 1992; Mandelkow et al., 1992; Vulliet et al., 1992; Baumann et al., 1993). In addition, a cdc2-like kinase and the MAP kinase ERK2 were shown to associate with NFTs in AD brains by immunohistochemical analysis (Trojanowski et al., 1993; Liu et al. 1995). Tau is also phosphorylated by second messenger kinases including cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and Ca²⁺/calmodulin-dependent kinase (CaM kinase II) (Baudier and Cole, 1987; Correas et al., 1992; Litersky and Johnson, 1992). Other kinases such as p110<sup>марк</sup>, casein kinase I and II, phosphorylase kinase and DNA-dependent kinase have also been implicated in the phosphorylation of tau (Steiner et al., 1990; Wu et al., 1993; Drewes et al., 1995; Paudel, 1997a). Interestingly, GSK-3β is the only kinase known to phosphorylate tau <i>in vivo</i> as demonstrated by co-transfection of tau and this kinase into COS, 3T3, and CHO cells (Lovestone et al., 1996; Wagner et al., 1996). Upon <i>in vitro</i> phosphorylation, tau can shift in electrophoretic mobility in a manner similar to that seen with PHF-tau. PHF-tau typically migrates as three distinct bands of 60, 64, and 68 kDa on SDS-PAGE (Flament and Delcourte, 1989; Lee et al., 1991). Furthermore, <i>in vitro</i> phosphorylated tau reacts with antibodies to specific phosphoepitopes found present on PHF-tau (Figures 9 and 10). Thus, <i>in vitro</i> analysis of the phosphorylation state of tau has shed some light on the possible players involved in the hyperphosphorylation of PHF-tau, the sites important for modulating tau-MT interactions, and potential signaling pathways involved in AD.
This dissertation addresses one hypothesis regarding the etiology of AD, focusing on the hyperphosphorylation of tau. This hypothesis states that the reactivation of a fetal-like and/or mitotic kinases are occurring in selected pyramidal neurons (Kanemaru et al., 1992; Brion et al., 1993; Goedert et al., 1993). Previous results have shown that other neuronal MAPs, such as MAP1B, contain mitosis specific phosphoepitopes and that these epitopes are expressed in fetal brain and PC12 cells during differentiation and neurite outgrowth.

Chapter 1 is focused on determining if similar mitosis-associated phosphoepitopes appeared in AD. Sections of hippocampal tissue were stained for immunoreactivity with the MPM2 antibody, which recognizes mitotic phosphoepitopes. The MPM2 mitotic phosphoepitope antibody, and the AT8 PHF-tau antibody, were used in experiments to stain both AD and control brain samples. In addition, PHFs isolated from AD brains were analyzed for reactively with both antibodies. The MPM2 antibody specifically reacted with tau in the isolated PHF fraction, but not normal adult tau or tau obtained from fetal and adult rat brains. These results indicate that MPM2 recognized a phosphoepitope normally associated with mitosis that is also present on PHF-tau and may be specific for the disease state.

Chapter 2 is focused on the use of an in vitro phosphorylation assay to examine the temporal sequence of tau phosphorylation. A crude bovine brain
extract was utilized as the kinase source to phosphorylate recombinant human tau. Analysis of the various phosphoisoforms was carried out with a series of antibodies to both phosphorylation dependent and independent sites. The MPM2 antibody recognized phosphoisoforms of tau that migrated on polyacrylamide gels between the fully dephosphorylated and phosphorylated tau isoforms reactive with the BT2 and AT8 antibodies, respectively. The MPM2 epitope on tau was also more resistant to dephosphorylation than the AT8 epitope. Both soluble and microtubule-associated kinase activities were responsible for phosphorylating the AT8 epitope, whereas it appeared that only the soluble kinases were able to phosphorylate tau on the MPM2 epitope. Comparison of fetal and adult brain extracts also revealed potential variations in tau kinase activities. These results indicate that the MPM2 and AT8 phosphoepitopes on tau are distinct and regulated by different kinase and phosphatase activities.

In chapter 3, a series of experiments addressing the location of the MPM2 epitope on tau were carried out. First, phosphorylation of two recombinant tau isoforms either lacking one or both of the N-terminal inserts were analyzed for reactivity with the MPM2 antibody. This was followed by determination of two potential MPM2 sites on tau based upon the MPM2 epitope model. The potential sites on tau were tested for reactivity with the MPM2 antibody using deletion mutants of full-length recombinant human tau.
Mutations
670/671 692 716 717

Extracellular

NH₂ COOH

Aβ fragments

Membrane

Sites of Proteolytic Processing

Normal APP processing

soluble APP 3 kDa 7 kDa

NH₂ COOH

α-secretase γ-secretase

Aβ peptide formation

truncated soluble APP Aβ peptides 7 kDa

NH₂ COOH

β-secretase γ-secretase

Structure and Processing of the β-amyloid precursor protein (APP)

FIGURE 1
FIGURE 2

Structural diagram of Presenilin proteins
CA1, CA2, CA3, CA4: regions of hippocampus
DG: dentate gyrus
SB: subiculum
F: fomix

FIGURE 3
Diagram of the hippocampus
FIGURE 4

Paired helical filament (PHF) structural diagram
FIGURE 5

Microtubule associated proteins
FIGURE 6
Tau isoforms
| Human  | MAEPRQEFEV | MEDHAGTYGL | GDRKDQGGYT | MHQDQEGDTD | 40 |
| Rat    | MAEPRQEFDT | MEDQAG.... | ........DYT| MLQDQEGDM | 29 |
| Human  | AGLKESPLQT | PTEDGSEEPP | SETSDAKSTP | TAEDVTAPLV | 80 |
| Rat    | HGLKESPPQP | PADDGSEEPP | SETSDAKSTP | TAEDVTAPLV | 69 |
| Human  | DEGAPGKQAA | AQPHTEIEPP | TTAEEAGIGD | TPSLEDEAAG | 120 |
| Rat    | EERAPDKQAT | AQSHTEIEPP | TTAEEAGIGD | TPNMEDQAAG | 109 |
| Human  | HVTQARMVSK | SKDGTGSDDK | KAKGADGKTK | ..IATPRGAAPP | 160 |
| Rat    | HVTQARVAGV | SKDRTGNDEK | KAKGADGKTG | AKIATPRGAATP | 151 |
| Human  | GQKQANATR  | IPAKTPPPAPK | TPPSSGEPPK | SGDRSGYSSP | 200 |
| Rat    | GQKGTNSATR | IPAKTTSSPK | TPPGSGEPPK | SGERSGYSSP | 191 |
| Human  | GSPGTPGSR | RTPSLPTPPT | REPKKVAVVR | TPPKSPSSAK | 240 |
| Rat    | GSPGTPGSR | RTPSLPTPPT | REPKKVAVVR | TPPKSPSASK | 231 |
| Human  | SRLQTAPVPM | PDLKNVKURI | GSTENLKHQP | GGGKVQIINK | 280 |
| Rat    | SRLQTAPVPM | PDLKNVRISKI | GSTENLKHQP | GGGKVQIINK | 271 |
| Human  | KLDLSNQVK | CGSKDNIKHV | PGGGSVQIVY | KPVDLSKVT | 320 |
| Rat    | KLDLSNQVK | CGSKDNIKHV | PGGGSVQIVY | KPVDLSKVT | 311 |
| Human  | KCGSLGNIHH | KPGGGQVEVK | SEKLDFKDRV | QSKIGSLDNI | 360 |
| Rat    | KCGSLGNIHH | KPGGGQVEVK | SEKLDFKDRV | QSKIGSLDNI | 351 |
| Human  | THVPGGGNKK | IETHKLTFRF | NAKAKTDHG | EIVYKSPVVS | 400 |
| Rat    | THVPGGGNKK | IETHKLTFRF | NAKAKTDHG | EIVYKSPVVS | 391 |
| Human  | GDSRPHLSN | VSSTGSIDMV | DSPQLATLAD | EVSASLAKQGL | 441 |
| Rat    | GDSRPHLSN | VSSTGSIDMV | DSPQLATLAD | EVSASLAKQGL | 432 |

Lines indicate alternatively spliced sequences
Shaded amino acids: N-terminal inserts
Bold amino acids: Microtubule binding domains

**FIGURE 7**
 Tau amino acid sequence
FIGURE 8

Tau phosphorylation sites
<table>
<thead>
<tr>
<th>PHF-tau</th>
<th>Fetal-tau</th>
<th>GSK3b</th>
<th>cdk5</th>
<th>GSK3a</th>
<th>MAPK</th>
<th>PK 35/41</th>
<th>P110(марк)</th>
<th>PKA</th>
<th>PKC</th>
<th>CaM kinase</th>
<th>GSK3a</th>
<th>TTK</th>
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GSK = Glycogen synthase kinase; MAPK = MAP kinase or ERK; cdk5 = cyclin-dependent kinase; PKA = cAMP-dependent kinase; PKC = Calcium dependent kinase; CaM kinase = Calcium/calmodulin dependent kinase; TTK = Tau/tubulin kinase

FIGURE 9
Table of Tau protein kinases

42
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope</th>
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<tr>
<td>N-term</td>
<td>synthetic peptide: 1-15 aa</td>
</tr>
<tr>
<td>Alz50</td>
<td>discontinuous requires: 1-18 aa and sequences in MT repeats</td>
</tr>
<tr>
<td>TAU1</td>
<td>dependent on dephosphorylation: 189-207 aa</td>
</tr>
<tr>
<td>BT2</td>
<td>194-198 aa containing RSGYS sequence</td>
</tr>
<tr>
<td>pSer199</td>
<td>synthetic phosphopeptide 194-205 aa, phosphate on Ser 199</td>
</tr>
<tr>
<td>AT8</td>
<td>phosphorylated Ser 202 and Thr 205</td>
</tr>
<tr>
<td>AT10</td>
<td>phosphorylated Thr 212 and Ser 214</td>
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<td>PHF27</td>
<td>phosphorylated Thr 231 and Ser 235</td>
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<td>pSer262</td>
<td>synthetic phosphopeptide 257-267 aa, phosphate on Ser 262</td>
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<td>T3P</td>
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<tr>
<td>pSer396</td>
<td>synthetic phosphopeptide 390-401 aa, phosphate on Ser 396</td>
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<tr>
<td>PHF1</td>
<td>phosphorylated Ser 396 and Ser 404</td>
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<tr>
<td>C-term</td>
<td>synthetic phosphopeptide 420-430 aa</td>
</tr>
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</table>

**FIGURE 10**

**Tau antibodies**
CHAPTER 1

Alzheimer's Disease Neurofibrillary Tangles Contain Mitosis Specific Phosphoepitopes

INTRODUCTION

Amyloid-rich senile plaques and neurofibrillary lesions are two of the neuropathological hallmarks of Alzheimer's disease (AD). The neurofibrillary lesions consist of neurofibrillary tangles (NFTs), neuropil threads, and senile plaque neurites. The principal structural component of neurofibrillary lesions are paired helical filaments, PHFs (Kidd et al., 1964), which are largely composed of the microtubule associated protein (MAP) tau (Grundke-Iqbal et al., 1986a). PHFs are found in a select population of neurons including the pyramidal cells of the CA1 region of the hippocampus, layers II and IV of the entorhinal cortex, and layers III and V of the association cortex (Pearson et al., 1985; Ihara, 1988; Wischik et al., 1995). The extent and severity of disease correlates with the number and distribution of NFTs within the hippocampus and cortex.
In the adult brain, tau consists of six isoforms, whereas only one isoform is expressed in the fetal brain (Goedert et al., 1989a; 1989b; Kosik et al., 1989). PHF-tau is composed of all six tau isoforms (Goedert et al., 1992b), however, PHF-tau differs from normal tau in that: 1) PHF-tau is highly insoluble (Crowther and Wischik, 1985); 2) PHF-tau can be distinguished from normal tau by certain phosphorylation dependent antibodies (Grundke-Iqbal et al., 1986b; Wood et al., 1986; Brion et al., 1991; Biernat et al., 1992); and 3) PHF-tau is retarded on sodium dodecyl sulfate (SDS) gels migrating as three bands of 60, 64, and 68 kDa (Flament and Delacourte, 1989; Lee et al., 1991). All of these characteristics are believed to be due to the hyperphosphorylation of PHF-tau. In addition, PHF-tau is unable to bind microtubules indicating that abnormal phosphorylation leads to a decrease in its affinity for microtubules (Bramblett et al., 1993). The phosphorylation of PHF-tau is considered to be involved in degeneration of pyramidal neurons in AD, possibly through a mechanism involving destabilization of microtubules (Wischik et al., 1995). Some of the phosphorylation sites present in PHF-tau have also been shown to be phosphorylated in normal fetal tau (Kanemaru et al., 1992; Brion et al., 1993; Goedert et al., 1993; Hasegawa et al., 1993; Kenessey and Yen, 1993; Watanabe et al., 1993). Taken together, these observations suggest that fetal-like conditions are being reactivated in the AD brain.

Other neuronal MAPs such as MAP2 and MAP1B have been shown to associate with NFTs in brains of AD patients (Yen et al., 1987; Hasegawa et al., 1993).
1990; Takahashi et al., 1991; Ulloa et al., 1994). Normally, levels of MAP1B protein are highest during fetal and early postnatal development, and it is located predominantly in the developing axon. Although MAP2 and tau are also expressed during development, MAP1B is the only MAP consistently found in extending neurites (Müller et al., 1994). Also, MAP1B is the major MAP found in neurites of cultured PC12 cells (Brugg and Matus, 1988), and it becomes phosphorylated during differentiation and neurite outgrowth in these cells (Aletta et al., 1988). Phosphorylated forms of MAP1B are also present in hippocampal pyramidal cells (Fischer and Pomano-Clarke, 1991) and both fetal and adult rat brain (Díaz-Nido et al., 1990).

Previous data has revealed that MAP1B present in isolated porcine brain microtubules contains mitosis specific phosphopeptides identified by the MPM2 monoclonal antibody (Vandré et al., 1986). We have also shown that MAP1B present in fetal and adult rat brains, as well as in differentiated PC12 cells, express these phosphopeptides (D.D. Vandré et al., unpublished observations). The MPM2 antibody was prepared against a mitotic HeLa cell extract and recognizes a set of phosphoproteins present in mitotic cells (Davis et al., 1983; Vandré et al., 1991). Based upon previous results indicating that MAP1B was associated with PHFs, we examined whether pathological structures within hippocampal brain sections obtained from AD patients were recognized by the MPM2 antibody. Our immunohistochemical analysis revealed that the MPM2 epitope was expressed in
the neurofibrillary lesions of AD brains. Furthermore, analysis of isolated PHF preparations showed that the MPM2 staining was not due to the presence of MAP1B, but rather resulted from the MPM2 reactivity of PHF-tau. It is interesting that MPM2 did not recognize fetal or adult rat brain tau. Thus, the MPM2 antibody recognizes a specific AD phosphoepitope distinct from known phosphoepitopes that are also shared with fetal rat brain tau.
EXPERIMENTAL PROCEDURES

Antibodies

A panel of monoclonal antibodies to various MAPs were used for immunohistochemical staining of brain sections and Western blots. Two antibodies were used to identify all normal tau isoforms present in human, rat, and bovine brain, the TAU1 (Boehringer Mannheim, Indianapolis, IN) and BT2 (Biosource International, Camarillo, CA) antibodies. TAU1 recognizes a dephosphorylated epitope located between amino acids 192-204 (Szendrei et al. 1993). The BT2 antibody reacts with a tau epitope located at positions 194-198 containing the RSGYS amino acid sequence, and does not recognize PHF-tau. The antibody used to recognize PHF-tau was the AT8 antibody (Biosource International, Camarillo, CA), which recognizes an epitope having both phosphorylated residues at Ser 202 and Thr 205 (Goedert et al., 1995). In addition, AT8 has been shown to recognize phosphorylated forms of fetal tau (Goedert et al., 1993). The other neuronal MAP antibodies used in this study were raised against MAP1A, clone 1A1 (Amersham, Arlington Heights, IL); MAP2, clone MAP2.3 (Amersham, Arlington Heights, IL); and MAP1B, MAP5 antibody (Sigma Chemical Co., St. Louis, MO) and clone 1B4 (R. Vallee, Worcester Foundation for Experimental Biology, Shrewsbury, MA). The MAP2.3 antibody reacts with MAP2a and MAP2b, but not MAP2c. Two other antibodies were used which recognize MAP phosphoepitopes, 1B3 and MPM2. The 1B3 monoclonal antibody (R. Vallee, Worcester Foundation for
Experimental Biology, Shrewsbury, MA) recognizes a common phosphoepitope present on both MAP1A and MAP1B. The MPM2 antibody (J. Kuang, M.D. Anderson Hospital, Houston, TX) reacts with a distinct class of phosphoproteins abundant in mitotic cells, including MAP1A and MAP1B, that are phosphorylated at the onset of mitosis (Vandré et al., 1991).

**Immunohistochemical assays**

Human hippocampal brain samples were obtained from the Department of Pathology, Neuropathology Laboratory at the Ohio State University. Samples were collected approximately 8-10 hours postmortem from clinically diagnosed AD, elderly, and younger control patients. The criteria for classification of the brain samples as AD was according to the protocol of Mirra et al. (1993). The samples were formalin fixed and then either paraffin-embedded or frozen in liquid nitrogen. Before freezing, the tissue was incubated overnight in 0.1 M phosphate buffered saline (PBS) containing 20% sucrose at 4°C. Tissue sections were cut either at 10-15 μm using a cryotome or at 5μm using a microtome depending on sample preparation. The frozen and paraffin sections were stored at either -70°C or room temperature respectively until use. Immunocytochemical staining was carried out with modifications to the previously described procedures (Osborn and Isenberg, 1994). First, the endogenous peroxidase activity was blocked in the tissue sections by incubation in methanol containing H₂O₂ for 30 minutes at room temperature. Next, the sections were washed with PBS for 5 minutes and then blocked for 1 hour.
with 4% normal goat serum at 37°C. The blocked sections were drained and incubated with primary antibody at 37°C for another hour. Following the primary antibody incubation, the sections were washed with PBS three times for 5 minutes each. The secondary antibody, goat anti-mouse (Kirkegaard & Perry Laboratories, Gaithersburg, MD) coupled to peroxidase, was added to the sections and incubated at 37°C for 30 minutes. Finally, the sections were developed using 3, 3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and counterstained with hematoxylin following three rinses with PBS and one rinse with 50 mM Tris buffer containing 0.1 M NaCl (pH 7.4).

**Tau isolation from fetal and adult rat brain**

Normal rat tau was examined in fetal and adult rat brain samples prepared by either of two methods. Whole brain homogenate samples were prepared based upon the protocol of Drewes et al. (1992). Briefly, the rat brains were homogenized at 4°C in buffer A [25 mM Tris-HCl (pH 7.4) containing 25 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, the phosphatase inhibitors 1 μM okadaic acid, 50 mM NaF, and 1 mM Na₃VO₄, and the protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml each leupeptin, pepstatin, and aprotinin]. The samples were centrifuged at 100,000 g for 30 minutes at 4°C, and the supernatant was removed and stored at -70°C until use in immunoblot assays. For further enrichment of tau protein, a procedure slightly modified from that of Brion et al. (1993) was used. Brains were removed from newborn and adult rats and stored at -70°C before tau
purification. The tissue samples (0.5 g/ml) were homogenized at 4°C in buffer H [50 mM Tris-HCL (pH 7.4), 10 mM EDTA and inhibitor cocktail consisting of the protease inhibitors 1 mM PMSF, 25 μg/ml leupeptin, and 25 μg/ml pepstatin, and the phosphatase inhibitor 10 μM okadaic acid]. The homogenized samples were centrifuged at 30,000 g for 20 minutes at 4°C. The supernatant was collected and brought up to 0.75 M NaCl and 2% (v/v) 2-mercaptoethanol. The samples were placed on ice for 15 minutes and then heated at 100°C for 5 minutes, followed by centrifugation at 22,000 g for 30 minutes at 25°C. The heat-stable supernatant containing tau and MAP2 was removed and stored at -70°C before use in immunoblot analysis.

**PHF-tau isolation**

A PHF-tau sample was purified from the right temporal lobe of a pathologically confirmed AD patient (a 67-year-old woman) based on previous reported protocols (Greenberg and Davies, 1990; Ksiezak-Reding et al., 1990; Goedert et al., 1992b). The brain tissue was obtained from the Cooperative Human Tissue Network. The samples were collected 10-14 hours postmortem and snap frozen before isolation. The tissue (29.0 g) was homogenized in 10 volumes (w/v) cold buffer C (10 mM Tris, 1 mM EGTA, 0.8 M NaCl, and 10% sucrose, pH 7.4) at 4°C. All further steps were done at 4°C unless noted. The homogenates were centrifuged at 20,000 g for 20 minutes. The supernatant, PHF.s1, was removed and placed on ice. The pellet was resuspended, rehomogenized in 10 volumes of
buffer C, and then centrifuged as before. A second supematant was removed, and both supematants were combined and adjusted to 1% (w/v) N-lauroylsarcosine. The adjusted sample was incubated at 37°C for 2 hours with gentle shaking. After incubation, the sample was centrifuged at 60,000 g for 30 minutes at room temperature. The pellets containing PHF-tau were collected, resuspended in approximately 2 ml of 50 mM Tris-HCl (pH 7.2), and stored at -70°C. The final supematant, PHF.s3, depleted of PHFs, was also saved for immunoblot analysis.

**Dephosphorylation of tau proteins**

Prior to Western blot analysis, dephosphorylation of PHF-tau was accomplished by modifications to a previously reported protocol (Goedert et al., 1992b). In brief, PHF-tau was treated with 4 M guanidine hydrochloride for 1 hour at room temperature with gentle stirring to denature the PHFs. The sample was dialyzed overnight at 4°C against 50 mM Tris-HCl (pH 8.0) plus 0.1 mM EDTA. After dialysis, the sample was adjusted to 1 mg/ml PMSF and treated with 400 U/ml calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) at 37°C overnight. The dephosphorylated PHF-tau was snap-frozen in liquid nitrogen, lyophilized, and resuspended in 50 mM Tris-HCl (pH 7.4) plus 10 mM EDTA. Sample buffer was added to the dephosphorylated PHF-tau and the material was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE).
Western blotting

The rat brain homogenates, normal rat tau, PHF-tau, and dephosphorylated PHF-tau were electrophoresed on 7% or 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes as described (Vandré et al., 1986). See Blue™ Pre-stained standards (Novex Experimental Technology, San Diego, CA) were used as the molecular weight markers. The nitrocellulose membranes were blocked with 10% normal horse serum overnight at room temperature. Primary antibodies diluted in blocking solution were incubated for 2 hours at room temperature, followed by a 1 hour incubation with goat anti-mouse immunoglobulins conjugated to peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The blots were developed with 4-chloro-1-napthanol in Tris buffered saline.
RESULTS

Immunoreactivity of MPM2 antibody with rat neuronal MAPs

The MPM2 antibody has been shown to react with certain neuronal MAPs such as MAP1A and MAP1B (Bloom et al., 1984; Vandré et al., 1986; DeMey et al., 1987; Tombes et al., 1991), however, the recognition of tau protein or phosphorylated forms of tau by the MPM2 antibody has not been examined. Therefore, we determined whether fetal or adult rat brain tau was MPM2 reactive as determined by immunoblot analysis of whole rat brain homogenates (Figure 11) and partially isolated tau protein preparations (Figure 12). Several antibodies were used to identify the different neuronal MAPs and phosphorylated epitopes in each preparation.

In blots of the whole brain homogenate, the MPM2 antibody reacted with MAP1 (Figure 11A, arrow). MAP1A and MAP1B were not clearly resolved on these 7% SDS acrylamide gels, but we have demonstrated that both proteins are MPM2 reactive (data not presented, Vandré et al. 1986). The MPM2 reactivity observed in the fetal fraction was primarily due to MAP1B, because only extremely low levels of MAP1A were present in the fetal brain samples. Both MAP1 isoforms were present in the adult brain; however, most of the MPM2 reactivity was due to the MAP1B protein (data not presented). The MPM2 antibody did not stain any low molecular weight (MW) bands in these homogenates. Also, the MAP2.3
immunoblot (Figure 11A, lanes 5 and 6) confirmed the presence of MAP2 in both fractions; however, the MAP2.3 immunoreactive bands migrated at a slightly lower MW than the bands recognized by MPM2. These results indicated that MAP2 does not contain the MPM2 epitope. The 1B3 antibody (Figure 11A, lanes 3 and 4), which recognized a phosphoepitope common to both forms of MAP1, was also tested and reacted with the MAP1 present in fetal and adult brain. In addition, tau protein was present in both fetal and adult brain homogenates as indicated by the BT2 immunoblot (Figure 11B). As expected, a broad doublet around 50 kDa was observed in the fetal fraction, whereas the adult tau migrated as 5-6 evenly spaced bands between 50-67 kDa. Our BT2 blot was consistent with previous reports regarding tau expression in fetal and adult brains (Goedert et al., 1989; Kosik et al., 1989). Thus, it appeared that the MPM2 antibody did not react with either fetal or adult forms of tau.

The lack of MPM2 reactivity with the tau bands could have been due to the low concentration of phosphorylated tau in the whole brain homogenates. To address this possibility, we partially isolated tau from both fetal and adult rat brains, and analyzed these samples on Western blots. Again, both the fetal and adult rat samples showed no MPM2 reactivity with the isolated tau bands (Figure 12). The MPM2 antibody reacted with several high MW bands in the isolated tau fractions, but these are most likely degradation products of MAP1A or MAP1B. Also, a faint band at approximately 50 kDa in the fetal fraction (Figure 12, lane 3) was
recognized by MPM2. However, this band does not correspond to the fetal tau bands recognized by either TAU1 or BT2 antibodies, and it is likely to be a MAP1A or MAP1B degradation product as well. The PHF-tau antibody AT8 recognized only fetal tau but not adult tau (Figure 12), as has been previously reported (Goedert et al., 1993). The two AT8 reactive fetal bands migrated at a positions similar to those of the fetal tau bands recognized by the BT2 and TAU1 antibody. The multiple tau isoforms present in the adult brain samples as detected by both the TAU1 and BT2 antibodies were not recognized by the MPM2 or AT8 antibodies. Thus, these results confirm that the phosphoepitopes identified by the MPM2 (Figures 11 and 12) and 1B3 (Figure 11; data not presented) antibodies are not present on normal tau despite the elevated phosphorylation state of fetal tau.

Distribution of antibody staining in Alzheimer's disease brain sections

Phosphorylated MAPs such as MAP1B have been reported to be associated with NFTs (Hasegawa et al., 1990; Takahashi et al., 1991; Ulloa et al., 1994). To determine whether phosphoepitopes such as those recognized by the MPM2 antibody are associated with AD, we screened a series of brain sections for immunoreactivity with MPM2. Figure 13 contains a list of the samples used in these immunohistochemical experiments. We compared brain sections from the hippocampus of clinically diagnosed AD (n=10) patients with elderly (n=6) and younger (n=2) controls. All the AD brain sections were stained by both the MPM2 monoclonal antibody and the PHF-tau specific AT8 antibody (Figure 14). MPM2
staining was localized to the cell bodies of selected pyramidal neurons and plaque-like structures resembling senile plaques (Figure 14A). Furthermore, the MPM2 staining was similar to that obtained with the AT8 antibody on sections from the same individuals (Figure 14B). We noted, however, that there was some individual variability in the MPM2 staining of lesions and intensity between cases (data not presented). This difference in staining intensity could have some implication in reflecting the severity of disease. For example, the most highly reactive area of the hippocampus was the CA1 region which indicates a stage IV or higher progression of disease. Some of the elderly controls showed rare examples of NFT staining within selected pyramidal neurons (data not presented). This staining pattern was most likely due to the normal aging process, because the younger control sections showed no MPM2 reactivity. Previous experiments with phosphospecific antibodies, such as T3P, have also shown occasional staining of hippocampal neurons in age-matched controls (Lee et al., 1991).

MPM2 antibody stained several structures characteristic of the pathological lesions associated with AD brains, including NFTs (Figure 15A), senile plaques (Figure 15B), neuropil threads (Figure 15C, arrow), and pyramidal neurons undergoing a vacuolar-like degeneration (Figure 15D, arrow). A similar granular staining pattern in the cytoplasm of selected hippocampal neurons of AD brain samples has been observed previously using an antibody against a phosphoepitope present on MAP1B (Ulloa et al., 1994). In addition to the granular or vacuolar
cytoplasmic staining observed in some neurons, the MPM2 antibody clearly stained filamentous structures within the cell body that resembled PHFs (Figure 15A). To determine if the MPM2 antibody was recognizing the same set of neurons as the PHF-tau specific AT8 control antibody, staining of serial sections of the brain samples were compared. MPM2 and AT8 staining was demonstrated to colocalize to the same set of pyramidal neurons (Figure 16) and senile plaques (Figure 14; data not presented). The staining pattern of both antibodies was also restricted to the cytoplasm of the reactive neurons and was excluded from the nucleus. These results suggested that the MPM2 antibody could be reacting with either a phosphorylated neuronal MAP associated with NFTs or PHF-tau itself.

To examine whether other antibodies that recognized MAP1 gave staining patterns similar to that observed with MPM2 in AD sections, we compared the staining of MAP1 antibodies including the 1A1, 1B4, and 1B3 antibodies. The MAP1A antibody (Figure 17A) showed staining of the cell bodies of pyramidal neurons (arrowhead) and their dendrites. Also, the cytoplasm of the granule cells of the dentate gyrus was stained by the 1A1 antibody (Figure 17A, arrow). In contrast to the MAP1A staining pattern, the 1B4 antibody (Figure 17B) reacted only weakly with the cytoplasm of the pyramidal neurons (arrowhead) and did not stain dendritic processes. The granule cells (Figure 17B, arrow) were also not stained by the 1B4 antibody. The MAP phosphoepitope specific 1B3 antibody also had a staining pattern that was distinct from MPM2 (Figure 17C). Both the pyramidal
neurons (arrowhead) and granule cells (arrow) were reactive, but it appeared that the antibody localized to the nucleus of the granule cells. The level of staining with the 1B3 antibody was extremely high even with low concentrations of the antibody. All of the specific MAP1 antibodies used appeared to stain the majority, if not all, of the pyramidal neurons, whereas the MPM2 and AT8 antibodies only stained those pyramidal neurons exhibiting degenerative pathology (Figure 16). These results suggested that the MPM2 antibody was not recognizing the general distribution of MAP1A and MAP1B. Thus, MPM2 was recognizing a distinct population of pyramidal neurons that contained NFTs and a phosphoepitope that was present on a subset of MAP1 proteins, associated with PHF-tau, or associated with another unique phosphoprotein present within AD neurons.

**MPM2 immunoreactivity of isolated PHFs**

Previous reports have demonstrated that many of the sites phosphorylated on PHF-tau are also phosphorylated in normal fetal tau (Kanemaru et al., 1992; Brion et al., 1993; Goedert et al., 1993; Hasegawa et al., 1993; Kenessey and Yen, 1993; Watanabe et al., 1993). The Western blot analysis of rat neuronal MAPs (Figures 11 and 12) showed that the MPM2 epitope was not found on either the adult or fetal phosphorylated tau isoforms. However, in AD brain tau is known to be abnormally phosphorylated at additional sites distinct from those present on fetal tau. It was possible, therefore, that the MPM2 antibody recognized an AD specific tau phosphorylation site.
In order to determine if MPM2 was reacting with NFT-associated MAP1B or PHF-tau, we isolated PHF-tau (Figure 18) from a pathologically diagnosed AD patients. A series of Western blots were carried out on the samples obtained during the isolation of PHFs to follow MAP1B and tau fractionation. Normal adult tau fractionated into the supernatant or soluble AD brain fractions, respectively, PHF.s1 and PHF.s3 (see Experimental Procedures and Figure 18), and was identified by the BT2 antibody as four to five evenly spaced bands migrating between 52-68 kDa (Figure 19). This result is consistent with previous reports (Ksiezak-Reding et al., 1992; Hasegawa et al., 1993). Also, some lower MW bands were identified by the BT2 antibody in the whole brain supematant fraction. In addition to normal adult tau, MAP1B was also present in the soluble fractions, but was not detected in the PHF pellet (Figure 19). The MPM2 antibody reacted with MAP1B in the soluble fractions, but not in the final PHF pellet. In contrast, several lower MW proteins ranging from 48 to 70 kDa were detected in the final PHF pellet fraction by the MPM2 antibody (Figure 19). The PHF-tau specific antibody, AT8, also stained a series of bands between 48-77 kDa in the final pellet fraction (Figure 19). The AT8 antibody did not react with proteins present in any other fraction indicating that the final pellet fraction was highly enriched for PHF-tau. The three high MW bands detected by AT8 between 57-70 kDa are similar to the three major PHF-tau polypeptides of 60, 64, and 68 kDa that have been identified in similar PHF preparations (Wolozin et al., 1986, 1988; Greenberg and Davies, 1990; Ksiezak-Reding et al., 1990). The lower molecular weight BT2 and AT8 bands probably
represent proteolytic degradation products of PHF-tau and normal tau, respectively, that were present in the AD brain samples. Previous data supports this conclusion, because PHF-tau digested by trypsin or chymotrypsin yields 40-45 kDa and 56-66 kDa fragments, respectively (Ksiezak-Reding et al., 1990). In addition, similar lower MW bands have been reported in normal tau and PHF-tau preparations (Ksiezak-Reding et al., 1988, 1990; Alonso et al., 1996). It is interesting that the AT8 reactive bands seemed to correspond to the low MW bands present in the MPM2 blot (Figure 19).

To analyze further the relationship between the low MW MPM2 reactive bands, and the PHF-tau polypeptides recognized by AT8, different brain samples were used to isolate PHF-tau (Figure 20). Again, the PHF-tau fraction showed reactivity with both the AT8 and MPM2 antibodies (Figure 20). The AT8 antibody clearly recognized the 60, 64 and 68 kDa PHF-tau polypeptides (bracket) as well as some lower MW bands. MPM2 antibody reacted strongly with the PHF-tau polypeptides in one sample but showed preferential staining of the 68 and 64 kDa polypeptides over the 60 kDa band in the second sample. Thus, this staining pattern could possibly indicate individual variability between patients. The MPM2 antibody also stained a subset of the lower MW PHF-tau bands that were also recognized by the AT8 antibody. As indicated above, these MPM2 reactive lower MW PHF-tau bands are most likely proteolytic degradation products of the three major PHF-tau polypeptides. It is reasonable that some degradation of the PHF-tau
bands occurred, because the autopsy samples were not collected for nearly 10 hours postmortem and protease inhibitors were not added to the buffers during isolation of the PHF-tau. Again, the MPM2 staining intensity of the degradation products appears to vary among different samples indicating possible variation between patients and/or sample collection. Overall, in comparison with the AT8 staining these results suggest that MPM2 may preferentially react with selected isoforms of PHF-tau.

To show that the MPM2 recognition required a phosphorylated PHF-tau epitope, the PHF-tau sample was first denatured and then dephosphorylated with alkaline phosphatase before immunoblot analysis. The MPM2 epitope on other neuronal MAPs is known to be phosphatase sensitive, and this is also true for the AT8 epitope on PHF-tau. The Western blot analysis of the dephosphorylated samples revealed that PHF-tau was completely dephosphorylated at both the AT8 and MPM2 epitopes (Figure 21). Concurrent with the loss of AT8 and MPM2 reactivity, the TAU1 epitope was exposed by the dephosphorylation of the PHF-tau sample. The dephosphorylated PHF-tau bands also shifted to a position on the gel of lower MW, and several of these bands appear to co-migrate with the normal tau identified by the BT2 antibody in supernatant fractions of AD brain homogenates (Figure 21). It is important to note that similar staining patterns were obtained after denaturation of the PHF sample by guanidine hydrochloride with the MPM2 or AT8 antibodies. Some variability in the staining intensity of the lower MW PHF-tau
bands was observed between different samples when stained with the MPM2 antibody. This may reflect different sensitivity of the MPM2 epitope to degradation in comparison to the AT8 epitope. Regardless, from these results it is clear that PHF-tau contains a specific phosphoepitope identified by the MPM2 antibody.
DISCUSSION

We have shown that the MPM2 antibody selectively stains neurofibrillary lesions of AD brains. The MPM2 antibody specifically reacted with NFTs, senile plaque neurites, and neuropil threads all of which contain PHF-tau. Little or no staining was observed in normal brain sections at similar antibody concentrations. The immunohistochemical staining pattern of the MPM2 antibody was similar to that of the PHF-tau specific antibody AT8. Both antibodies colocalized to the same pyramidal neurons and senile plaques. The MPM2 antibody recognizes MAP1B on immunoblots of fetal and adult rat brain but it did not recognize tau protein in either of these samples. Neuronal MAPs other than tau, such as MAP2 (Yen et al., 1987; Dammerman et al., 1989) and MAP1B (Hasegawa et al., 1990; Takahashi et al., 1991; Ulloa et al., 1994) have been shown to react with NFTs. We show that MPM2 also recognizes human MAP1B by immunoblot analysis of AD brain fractions. However, it does not appear that binding to MAP1B contributed to the MPM2 staining pattern we observed in AD brain sections, because MAP1B was not detected in the isolated PHF fraction. Immunoblot analysis of the PHF fraction showed that the MPM2 antibody recognized PHF-tau. Several fetal tau phosphorylation sites present in rat brain are also shared with human PHF-tau, including the AT8 epitope (Goedert et al., 1993). The MPM2 antibody, however, did not recognize phosphorylated fetal rat brain tau. Thus, the only tau species that we have shown to express the MPM2 mitosis specific phosphoepitope was that present
in isolated PHFs. These results indicate that the MPM2 epitope present on tau may be specific to the disease state, however, this will need to be confirmed using a comparative analysis of human fetal brain tau.

Previous *in vitro* data have shown that the tau protein can be phosphorylated by a several kinases including protein kinase A and C (Litersky and Johnson, 1992; Correas et al., 1992), casein kinase II (Steiner et al., 1990), Ca<sup>2+</sup>/ calmodulin kinase II (Baudier and Cole, 1987), glycogen synthase kinase-3, GSK-3, (Hanger et al., 1992; Mandelkow et al., 1992), mitogen-activated protein kinase, MAPK, (Drewes et al., 1992; Goedert et al., 1992a), p34<sup>cdc2</sup> kinase (Ledesma et al., 1992; Vulliet et al., 1992), and cdc2-like kinases (Baumann et al., 1993). Several of these kinases such as MAPK, GSK-3, and cdk5 are proline-directed kinases and phosphorylate tau on Ser-Pro and/or Thr-Pro sites, inducing an Alzheimer-like phosphorylation state. For instance, GSK-3 can phosphorylate recombinant tau produced in *E. coli* (Hanger et al., 1992; Mandelkow et al., 1992) and tau transfected into mammalian COS cells (Lovestone et al., 1994), generating several PHF-tau epitopes including the AT8 epitope. These three kinases are found in neurons and have also been shown to copurify with microtubules (Baumann et al., 1993). In addition, many of these kinases are known to be regulated during the cell cycle (Ledesma et al., 1992; Vulliet et al., 1992). Several proline-directed kinases have been implicated in
the regulation of cell cycle progression, suggesting that these or related cell cycle-dependent kinases may contribute to the abnormal phosphorylation of tau associated with AD.

The MPM2 epitope has not been defined biochemically, however, several MPM2 reactive phosphopeptides have been identified (Westendorf et al., 1994). A proline-directed phosphorylation site was present in each of these immunoreactive peptides. We have not identified the specific site recognized by the MPM2 antibody that is present on PHF-tau, but several of the known sites are proline-directed. Although the MPM2 epitope site on PHF-tau may be unique, it is also possible that it recognizes one of the previously identified phosphorylation sites. If this were the case, the MPM2 antibody may be a unique antibody probe for such a biochemically defined site.

It has recently been shown that tau protein becomes phosphorylated during mitosis in human neuroblastoma cell lines (Pope et al., 1994). In addition, tau transfected into Chinese hamster ovary (CHO) cells also shows increased phosphorylation levels in mitotic cells (Preuss et al., 1995). In both studies, the phosphorylated tau protein increased in apparent MW on acrylamide gels and gained reactivity with PHF specific antibodies. These results indicate that at least some of the phosphoepitopes associated with PHF-tau can be generated by kinase activity present in mitotic cells. Other MAPs, including MAP1B, have also been
shown to be phosphorylated during mitosis, and this phosphorylation is associated with their reactivity with the MPM2 antibody (Tombes et al., 1991; Vandré et al., 1991; Y. Choi and D.D. Vandré, unpublished observations). While this manuscript was in preparation, Vincent et al. (1996) reported on several new monoclonal antibodies raised against a preparation of PHFs. These antibodies were shown to recognize phosphoepitopes in a cell cycle-regulated fashion in cultured neuroblastoma cells. This study also showed that the MPM2 antibody recognized neurofibrillary lesions in AD brains; however, the MPM2 antibody did not react with a PHF preparation immunoaffinity purified with the Alz50 antibody. Thus, these authors concluded that the MPM2 antibody must be recognizing other phosphoproteins in AD brains. On the other hand, we have shown that at least some isoforms of PHF-tau share an MPM2 phosphoepitope that is common to other MAPs during mitosis. These differences may result from our isolation protocol for PHF-tau, which may not have selected for a subset of PHFs obtained by immunoaffinity purification with the Alz50 antibody (Vincent et al., 1996). Our results are consistent, however, with the demonstration that the MPM2 epitope, like other mitotic phosphoepitopes, is present on PHF-tau. The presence of the MPM2 mitosis associated epitope on tau in pyramidal neurons is unusual since these cells are clearly not mitotic. Therefore, selected neurons in the AD brain may have reactivated inappropriate developmental programs. As part of this reactivation, certain fetal-like pathways may have been initiated that eventually lead to the activation of mitosis-associated kinases.
Activation of mitosis-associated kinases may only be part of the reason that PHF-tau is phosphorylated on the MPM2 epitope in AD brains. The phosphatase(s) responsible for maintaining the dephosphorylation state of tau may also be inactivated in AD neurons. This possibility has recently been examined by Dupont-Wallois et al. (1995), using a neuroblastoma cell line treated with the phosphatase inhibitor okadaic acid. In these inhibitor treated cells, the hyperphosphorylation of tau was observed through the loss of TAU1 immunoreactivity and subsequent gain of AT8 and other PHF epitopes. In addition, the phosphorylated tau protein induced by the okadaic acid migrates similar to the 60 and 64 kDa PHF-tau polypeptides. It has also been shown that tau isolated immediately from fresh brain samples is phosphorylated and recognized by several PHF antibodies (Garver et al., 1994). The abundance of these phosphorylated tau isoforms is low, and is thought to be maintained at this level by phosphatase activity present in normal tissue. It is possible that MPM2 may also recognize a low-abundance fraction of normal adult human tau but that these isoforms are lost in the postmortem AD samples we have examined. In part, the abundance of phosphorylated tau may increase in AD brain through the inactivation of the normal phosphatase activities in degenerating cells. Perhaps in AD brains, both the AT8 and MPM2 epitopes are stable in PHF-tau under conditions of phosphatase deregulation. Both phosphatase-2A and -2B can rapidly dephosphorylate PHF-tau on several abnormally phosphorylated sites,
including that recognized by the AT8 antibody (Gong et al., 1994). Similarly, we show that the MPM2 epitope can be removed from PHF-tau by the action of alkaline phosphatase \textit{in vitro}.

Taken together, these observations suggest a dual involvement in the formation of PHF-tau between reactivation of mitosis-associated or related kinases and the inactivation of the respective phosphatases. The phosphorylation of the MPM2 epitope on PHF-tau could aid in identifying the kinase or kinase family responsible for the hyperphosphorylation of tau in AD. A clear understanding of the altered regulatory pathways affecting pyramidal neurons could lead to the development of therapeutic strategies for combating AD in the future.
Figure 11. Western blot analysis of fetal and adult rat brain homogenates.

Panel A: A 7% SDS-PAGE of fetal (odd lanes) and adult (even lanes) whole rat brain homogenates were used for immunoblot analysis. The arrow indicates the position of MAP1A and MAP1B. The antibodies used to probe the transfer were MPM2 at 1:2000 (lanes 1 and 2), 1B3 at 1:750 (lanes 3 and 4), MAP2.3 at 1:500 (lanes 5 and 6), MAP5 (lanes 7 and 8), and 1A1 at 1:500 (lanes 9 and 10). The MW markers from top to bottom are as follows: 250, 98, 64, and 50 kDa.

Panel B: A tau immunoblot of a 10% SDS-PAGE containing fetal (lane 1) and adult (lane 2) rat brain homogenates. BT2 antibody at 1:100 was used to stain the nitrocellulose transfer. The MW markers from top to bottom are as follows: 98, 64, 50, and 36 kDa.
FIGURE 11
Figure 12. Western blot analysis of isolated tau protein.

A 10% SDS-PAGE of isolated tau protein from fetal (odd lanes) and adult (even lanes) rat brains were used for immunoblot analysis. The antibodies used to stain the nitrocellulose transfer were BT2 at 1:100 (lanes 1 and 2), MPM2 at 1:1000 (lanes 3 and 4), TAU1 at 1:1000 (lanes 5 and 6), and AT8 at 1:500 (lanes 7 and 8). The MPM2 antibody does not react with fetal or adult isoforms of rat brain tau. The MW markers from top to bottom are as follows: 98, 64, 50, 36, and 30 kDa.
FIGURE 12
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Diagnosis: AD, Alzheimer’s disease; PD, Parkinson’s disease; DLB, Diffuse Lewy Body disease.
Reactivity: negative (-), occasional staining of random pyramidal cells within an entire hippocampal section; weak (+), scattered staining mainly in the subiculum and CA1 regions with occasional NFTs in CA2, CA3, and CA4 regions of the hippocampus; medium (++), staining throughout the subiculum, CA1 & CA2 regions and a few cells within CA4 region; and high (+++), staining throughout the subiculum and CA1-CA4 region. Lesions: SP, senile plaques; NFT, neurofibrillary tangles; and NT, neuropil threads.

FIGURE 13
Table of AD and normal cases
Figure 14. Immunohistochemical staining of Alzheimer's disease and normal brain sections.

The brain sections were prepared from the CA1 region of the hippocampus. AD brain sections from an 80-year-old female (A and B), and nondemented control brain sections from an 73-year-old female © were stained with the MPM2 antibody at 1:800 (A and C) or the AT8 antibody at 1:40 (B). The typical "flame cell" appearance of NFT-containing pyramidal cell neurons was observed with both antibodies in the AD brain samples. In addition, senile plaques were also stained by both antibodies (arrow). Only background levels of staining were present in control brain sections with the MPM2 antibody. Bar = 100 μm
Figure 15. MPM2 staining of neuropathological lesions in AD brain.

The MPM2 antibody (1:800) stained several pathological structures in the sections of the hippocampal tissue that are associated with AD. These include NFTs within pyramidal neurons (A), senile plaques (B), neuropil threads (arrows, C), and granulovacuolar degeneration (arrow, D). Bar = 25 μm
Figure 16. Colocalization of MPM2 and AT8 staining in serial sections of AD brain samples.

Sections were stained with the MPM2 antibody at 1:800 (A and C) or the AT8 antibody at 1:40 (B and D). The same pyramidal neurons are stained by both antibodies (compare A with B and C with D). Also note that only degenerating neurons are stained by each antibody whereas other neurons are not labeled (arrow). Bars = 25 μm
Figure 17. Immunostaining of AD brain sections with other neuronal MAP antibodies.

AD brain sections were stained with various MAP1 specific antibodies. Panel A: 1A1 at 1:200, Panel B: 1B4 at 1:200, and Panel C: 1B3 at 1:200. The arrow identifies granule cells of the dentate gyrus, and the arrowhead indicates the pyramidal neurons present in the hippocampus. The staining patterns obtained with these MAP antibodies was not the same as that observed with either the MPM2 of AT8 antibodies. In general, these MAP1 antibodies stained the granule cells within the dentate gyrus and all of the pyramidal cell bodies, whereas the MPM2 and AT8 antibodies did not. Bar = 100 μm
Homogenized tissue in 10 volumes (w/v) cold buffer C

Centrifuged homogenates and collected supernatant (s1) and pellet (p1)

Rehomogenized pellet (p1), centrifuged, and collected supernatant (s2)

Adjust combined supernatants to 1% N-lauroylsarcosine & incubated for 2 hr

Centrifuge sample and pellet containing PHF-tau

FIGURE 18
PHF purification scheme
Figure 19. Immunoblot analysis of isolated PHF preparation.

The fractions were separated on 10% SDS-PAGE with equal amounts of protein loaded per lane. Isolated PHF fractions with the first supernatant fraction, PHF.s1 (lane 1), final supernatant fraction, PHF.s3 (lane 2), and the PHF-tau pellet (lane 3). The antibodies used to stain the transfer were MAP5 at 1:500; MPM2 at 1:1000; AT8 at 1:500; and BT2 at 1:250. The arrow indicates the position of the MAP1B protein, which reacts with both the MAP5 and MPM2 antibodies. The MW markers are 98, 64, 50, 36, and 30 kDa.
FIGURE 19
Figure 20. Immunoblot of PHF-tau from separate AD brain samples.

Two different PHF-tau pellet fractions were isolated from separate AD brain samples and were analyzed by 10% SDS-PAGE followed by immunoblot. The concentrations of the MPM2 antibody and AT8 antibody were 1:1000 and 1:500, respectively. The bracket identifies the A68 or PHF-tau polypeptides which are reactive with both antibodies. The MW markers are 98, 64, 50, 36, and 30 kDa.
Figure 21. Dephosphorylation of PHF-tau.

Proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose before Western blot analysis. The samples correspond to PHF-tau (lane 1), denatured PHF-tau (lane 2), and dephosphorylated PHF-tau (lane 3). The blots were probed with the antibodies: MPM2 at 1:1000, AT8 at 1:500, and TAU1 at 1:1000. The MW markers are the same as in Figure 2. The reaction of PHF-tau with MPM2 and AT8 was sensitive to phosphatase treatment, and the dephosphorylated tau was identified by the TAU1 antibody. The BT2 blot (1:250) was of the first supernatant fraction, PHF.s1, identifying the normal tau. After dephosphorylation of the PHF-tau several bands identified by the TAU1 antibody comigrate with the normal tau.
FIGURE 21
CHAPTER 2

Generation and Characteristics of Tau Containing the Mitosis- and Alzheimer’s Disease-Associated MPM2 Phosphoepitope Following \textit{In Vitro} Phosphorylation

INTRODUCTION

The microtubule-associated protein (MAP) tau is the major structural component of paired helical filaments (PHF) in Alzheimer’s disease (Kidd, 1964; Grundke-Iqbal et al., 1986a) and other neurodegenerative disorders (Joachim et al., 1987; Baum et al., 1992; Smith and Lippa, 1995). Aggregates of PHFs and other proteins assemble within the cytoplasm of hippocampal pyramidal neurons to form neurofibrillary tangles, one of the neuropathological hallmarks of Alzheimer’s disease (AD). Six isoforms of tau are expressed in the adult brain whereas only one isoform is expressed during fetal development (Goedert et al., 1989a; 1989b; Kosik et al., 1989). In addition to tau being developmentally regulated by alternative mRNA splicing, the phosphorylation state and sites on tau vary among fetal, adult, and AD tau. The highest level of tau phosphorylation occurs in the AD brain and
is thought to induce self-association of tau into PHFs. All six adult isoforms of tau are both hyper- and persistently phosphorylated in AD brains (Grundke-Iqbal et al., 1986b; Goedert et al., 1992b).

The hyperphosphorylation of tau results in the inability of tau to bind and stabilize microtubules (Gustke et al., 1992; Bramblett et al., 1993; Alonso et al., 1994). In particular, PHF-tau is incapable of promoting in vitro assembly of microtubules unless previously dephosphorylated with alkaline phosphatase (Bramblett et al., 1993; Iqbal et al., 1994). Alterations in normal tau-microtubule interactions due to phosphorylation could lead to destabilization of the neuronal cytoskeleton and loss of normal axonal transport in affected neurons. The fetal isoform of tau is also heavily phosphorylated, but not to the same extent as PHF-tau. Several of the phosphorylation sites present on fetal tau are common to PHF-tau (Ihara et al., 1986; Kanemaru et al., 1992; Bramblett et al., 1993; Kenessey and Yen, 1993; Watanabe et al., 1993). The related pattern of tau phosphorylation in fetal and AD brains has led to the speculation that fetal-like phosphorylation events were reoccurring in AD hippocampal neurons. However, normal adult tau rapidly isolated from human biopsy samples is partially phosphorylated at several sites that were thought to be AD specific (Matsuo et al., 1994; Garver et al., 1994). Thus, not only the extent but also the persistence of phosphorylation at specific sites may be a factor in PHF-tau formation. Therefore, improper regulation of both kinase and phosphatase activities could be contributing causes of aberrant tau phosphorylation.
Several human neuroblastoma cell lines, including SHSY-5Y and LAN5, have been used as an in vivo model system to analyze the phosphorylation state of tau (Chiang et al., 1993; Pope et al., 1994; Smith et al., 1995; Tanaka et al., 1995; Shea and Fisher, 1996). Tau isoforms present in SHSY-5Y cells are partially phosphorylated on some AD sites, including Ser 199, Ser 202, Thr 231, Ser 396, and Ser 404 (Tanaka et al., 1995; Smith et al., 1995). Treatment of neuroblastoma cells with okadaic acid enhances the phosphorylation of tau, thus shifting tau from a fetal-like to an AD-like phosphorylation state (Dupont-Wallois et al., 1995; Chiang et al., 1993). Enhanced tau phosphorylation was established by 1) an increase in the molecular weight of the tau isoforms, 2) loss of a normal tau epitope, Tau-1, and 3) increased detection of AD epitopes, such as Alz50, PHF-1, and AT8. Furthermore, AD-like tau isoforms induced by okadaic acid treatment display a 50% reduced ability to co-assemble with bovine microtubules (Shea and Fischer, 1996). Thus, tau phosphorylation in neuroblastoma cells suggests the involvement of tau directed kinases and phosphatases similar to those present in adult brain.

Several kinases can phosphorylate tau in vitro including the cell cycle dependent kinase p34cdc2 (Ledesma et al., 1992; Vulliet et al., 1992). An in vivo study using SHSY-5Y cells demonstrates that during mitosis tau phosphorylation increases along with an increase in expression of the PHF-1 tau phosphoepitope, Ser 396/404 (Pope et al., 1994). PHF-1 immunoreactivity is most intense in cells during metaphase/anaphase indicating that cell cycle dependent kinases may
contribute to the hyperphosphorylation of tau in AD. Stable transfection of recombinant human tau into CHO cells shows that tau is partly phosphorylated at Ser 202, Thr 205, Thr 231, Ser 235, Ser 396, and Ser 404 during interphase and remains associated with microtubules (Preuss et al., 1995). Interestingly, tau becomes completely phosphorylated, shifts in molecular weight, and shows "AD-like" immunoreactivity during mitosis. The overall extent of phosphorylation of the transfected tau is elevated along with an increase in both PHF-1 and AT8 immunoreactivity. In addition, a substantial amount of tau is found disassociated from the microtubules and in the cytoplasm. Recently, a series of monoclonal antibodies raised against PHFs were shown to specifically stain mitotic neuroblastoma cells (Vincent et al., 1996). These antibodies also recognize isolated PHF-tau prior to dephosphorylation, in addition to the characteristic neurofibrillary lesions of AD brain tissue. However, these monoclonal antibodies do not react with normal tau isoforms, dephosphorylated PHF-tau, or the 55-57 kDa tau proteins in nocodazole-arrested cells. These findings further suggest that mitosis-associated or cell-cycle regulating kinases are involved in the hyperphosphorylation of tau and may play a role in AD pathogenesis. This aberrant phosphorylation of tau may result from the reactivation of developmental or mitotic pathways in adult neurons.

The MPM2 antibody, which was raised against a mitotic HeLa cell extract and recognizes a set of mitotic phosphoproteins (Davis et al., 1983; Vandré et al.,
1991), has also been shown to stain neurofibrillary lesions of AD brains (Kondratick and Vandré, 1996; Vincent et al., 1996). While we have shown by immunoblot analysis that hyperphosphorylated tau present in PHFs isolated from Alzheimer's tissue was recognized by the MPM2 antibody (Kondratick and Vandré, 1996; Chapter 1), PHF-tau isolated by immunoaffinity purification using the Alz50 antibody did not appear to be MPM2-reactive (Vincent et al., 1996). Thus, it was not clear whether all isoforms of PHF-tau contain the MPM2 epitope. Therefore it was not only necessary to determine whether tau could be phosphorylated at the MPM2 epitope site in vitro, but it was also important to examine the relationship between MPM2-reactive tau with tau containing other PHF-associated phosphoepitopes. We developed an in vitro phosphorylation system using recombinant human tau in order to 1) demonstrate the ability of brain kinases to phosphorylate the MPM2 epitope site, and 2) compare the pattern of tau reactivity between MPM2 and other PHF-tau phosphorylation site specific antibodies. Our kinase assay revealed that recombinant tau could be phosphorylated in vitro using a bovine brain kinase extract at the MPM2 phosphoepitope as well as other PHF phosphoepitopes including that recognized by the AT8 antibody. The phosphorylation pattern of the recombinant tau indicated that the MPM2 and AT8 epitopes are distinct both in terms of their temporal sequence of phosphorylation and their sensitivity to phosphatases.
Antibodies and reagents

The two monoclonal antibodies used to identify phosphorylated epitopes on tau were AT8 (Biosource International, Camarillo, CA, U.S.A.) and MPM2 (Upstate Biotechnology International). The AT8 antibody was directed against Alzheimer's PHFs and recognizes phosphorylated Ser 202 and Thr 205 (Goedert et al., 1995) on both fetal and PHF-tau. The MPM2 antibody was raised against a mitotic HeLa cell extract and recognizes a phosphoepitope common to a set of mitotic phosphoproteins (Davis et al., 1983; Vandré et al., 1991). In addition to recognizing mitotic phosphoproteins, the MPM2 antibody has been shown to react with MAPs (Bloom et al., 1984; Vandré et al., 1986; DeMey et al., 1987; Tombes et al., 1991; Vandré et al., 1991) including PHF-tau (Kondratick and Vandré, 1996). The BT2 monoclonal antibody (Biosource International, Camarillo, CA, U.S.A.) was used to recognize normal isoforms of tau, since it reacts with a tau epitope at positions 194-198 containing the RSGYS amino acid sequence.

Three additional phosphoepitopes on tau were identified using polyclonal antibodies raised against synthetic phosphopeptides. The three phosphoserine antibodies, pSer199, pSer262, and pSer396, were generated from the corresponding tau amino acid sequences: RSGYS(Sp)PGSPGT, KSKIG(Sp)TENLKC-amide, and AEIVYK(Sp)PVVSGC-amide, respectively. The
phosphopeptides were synthesized by Genosys Biotechnologies Inc. (The Woodlands, Texas) with the Ser 262 and Ser 396 peptides containing the addition of a C-terminal cysteine residue. The C-terminal cysteine residue was used for conjugation of the phosphopeptide to the carrier protein, Keyhole limpet hemocyanin (KLH) (CalBiochem, La Jolla, CA) via cross linking agent m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce, Rockford, IL) (Morishima-Kawashima et al., 1995; Garver et al., 1994). Prior to coupling the cysteine containing peptides to KLH, the KLH was maleimide activated using the Pierce Imject Activated Immunogen Conjugation kit. The third phosphopeptide containing Ser 199 was conjugated to KLH by cross linking free amino groups with glutaraldehyde (Bangalore et al., 1992; Coghlan et al., 1994). The immune sera was tested for reactivity against 1 μg/ml of KLH- conjugated or free peptides using an alkaline phosphatase ELISA.

The E. coli BL21(DE3) cells containing the htau40 cDNA clone was provided as a gift from Michel Goedert (Medical Research Council, Cambridge, U.K.). All chemical reagents were purchased for Sigma Chemical Company (St. Louis, MO).

**Kinase extract isolation**

A crude kinase source was obtained from bovine brain according to the previously published procedure (Biemal et al., 1992) with some modifications. The bovine brain was homogenized in an equal volume (w/v) of buffer A [10 mM Tris-
HCI, pH 7.2 containing 5 mM EGTA, 2 mM dithiothreitol (DTT) and a protease inhibitor cocktail of 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptin, aprotinin, and pepstatin A. The homogenized brain was centrifuged at 30,100 g for 30 minutes at 4°C and the supernatant was retained for a second cold spin at 100,000 g for 30 minutes. The high speed supernatant from the second spin was used as the kinase source and the protein concentration was determined using bicinchoninic acid protein assay (Pierce). The crude kinase extract was frozen with liquid nitrogen and stored at -70°C prior to use in phosphorylation experiments. In addition, kinase extracts were also isolated from both fetal (E19) and adult rat brains under the same conditions.

**Recombinant human tau expression and isolation**

The recombinant human isoform htau40 (Goedert et al., 1989) was expressed in *E. coli* and purified as previously described (Goedert and Jakes, 1990). Briefly, colonies of transformed BL21 cells were grown on TYE plates containing 100 μg/mL ampicillin. The TYE plates consist of 15 g/L agar, 10 g/L tryptone, 5 g/L yeast extract and 8 g/L NaCl. Individuals colonies of BL21 cells were suspended in 5 mL 2x TY medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.4) containing 100 μg/mL ampicillin and grown at 37°C overnight. Each 5 mL cell suspension was expanded by diluting in 250 mL fresh 2x TY medium containing ampicillin and grown to an OD between 0.6-1.0 at 600 nm at 37°C. The transformed cells are induced to express tau by adding 5.8 mL 20 mg/mL IPTG per
liter cells and allowed to incubate for another 2.5 hour at 37°C. After induction, the cells are harvested by centrifugation for 15-20 minutes at 7000 rpm and the cell pellets are frozen in liquid nitrogen prior to use. The frozen cell pellets are resuspended in 20 mL extraction buffer (50 mM PIPES, pH 6.9 containing 1 mM DTT, 100 μM EDTA, 100 μM PMSF, 1 μM leupeptin, and 1 μM pepstatin A). The cell suspension is sonicated on ice for a total of 20 minutes in intervals of 5 minutes sonication followed by 5 minutes of no sonication or until the cell suspension was translucent. Following sonication, the cells were centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was adjusted to 0.5 M NaCl prior to boiling for 10 minutes in water bath with constant stirring in order to separate the heat-labile proteins from tau. The heated supernatant was cooled on ice before centrifugation at 13,000 rpm for 15 minutes at 4°C. The tau enriched supernatant was obtained and dialyzed overnight in buffer A (20 mM PIPES, pH 6.9 containing 50 mM NaCl, 2 mM DTT, 1 mM EGTA, 1 mM MgSO₄, and 100 μM PMSF). Prior to applying the tau enriched sample on the Mono S HR 5/5 FPLC column, the dialyzed sample was filtered (0.22 μm) and the column was equilibrated with buffer A at a flow rate of 1 mL/minute. A NaCl gradient of 0-45% buffer B (buffer A plus 500 mM NaCl) was run over 45 minutes with purified htau40 eluting at approximately 200 mM NaCl. Peak fractions were analyzed for the presence of tau by coomassie staining of a 10% SDS-PAGE. The fractions containing the majority of tau were combined and concentrated using a Centricon-10 (Amicon Inc., Beverly, MA). Protein concentrations were determined using the bioinchoninic acid protein assay (Pierce).
In vitro phosphorylation of tau

The in vitro phosphorylation of recombinant tau was based on two previous protocols (Bramblett et al., 1993; Drewes et al., 1993). The phosphorylation reaction was carried out by adding 6.2 μg crude kinase extract to 9.0 μg htau40 in 50 μl phosphorylation buffer for 24 hours at 37°C. The phosphorylation buffer contained 40 mM HEPES (pH 7.2), 5 mM EGTA, 2 mM DTT, 2 mM ATP, 5 mM MgCl₂, 1 μM microcystin, 1 mM PMSF, and 20 μg/mL leupeptin, pepstatin A, and aprotinin. The reaction was stopped by incubating the sample for 5 minutes at 100°C in the presence of 5X sodium dodecyl sulfate (SDS) sample buffer. Parallel controls were done by omitting the kinase extract or immediately stopping the reaction with SDS sample buffer. In some cases, modifications of the phosphorylation buffer were utilized including the replacement of microcystin with okadaic acid or the removal of both phosphatase inhibitors. Multiple time points were obtained by removing aliquots from a single reaction mixture. All samples were run on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose for Western blot analysis.

Tubulin isolation

Purified microtubule protein was prepared from bovine brain tissue as previously described (Borisy et al., 1975; Solty and Borisy, 1985) through two cycles of polymerization/depolymerization. Briefly, the bovine cortex was homogenized in an equal volume (w/v) of cold 0.1 M Piperazine-N,N'-bis[2-
ethanesulfonic acid] (PIPES) buffer, pH 6.9 containing 0.1 mM MgSO₄, 0.1 mM GTP, 0.1 mM EGTA, and 0.1 mM PMSF and centrifuged at 25,900 g for 30 minutes at 40°C to remove cellular debris. The supernatant (CBS) was saved and 1.0 mM GTP was added prior to warm polymerization at 37°C for 35 minutes. The polymerized microtubules were pelleted in a 37°C centrifuge at 37,000 g for 30 minutes and both pellet (HₛP) and supematant (HₛS) fractions were saved for further analysis. The HₛP fraction was resuspended in cold 0.1 M 2-[N-Morpholino]ethanesulfonic acid (MES) buffer, pH 6.4 containing 0.1 mM MgSO₄, 1 mM GTP, 0.1 mM EGTA, and 0.1 mM PMSF and placed on ice for 30 minutes to depolymerize the microtubules. The depolymerized HₛP fraction was cold centrifuged for 30 minutes at 37,000 g to remove any cold stable material. The resulting cold supematant (CₛS) was retained and used for a repeated second cycle of polymerization/depolymerization. Protein concentrations were determined for each fraction using the bicinchoninic acid protein assay (Pierce).
RESULTS

Immunoreactivity of in vitro phosphorylated htau40

Several in vitro phosphorylation assays have been described that are capable of producing PHF phosphoepitopes on recombinant human tau (Billingsley and Kincaid, 1997). An in vitro phosphorylation assay using the longest isoform of human tau, htau40, and a crude bovine brain extract as a kinase source (Drewes et al., 1993; Bramblett et al., 1993), was used to determine if recombinant human tau could be phosphorylated on the MPM2 epitope. The phosphorylation assay was carried out in a buffer system containing 5 mM EGTA to inhibit endogenous protein phosphatase calcineurin and Ca\(^{2+}\)-dependent protein kinases present in the kinase extract. In addition, Ser/Thr protein phosphatase inhibitors were added to the phosphorylation buffer to block endogenous PP1 and PP2A. Phosphorylation of htau40 using the crude brain extract was analyzed by immunoblot with the MPM2 antibody (Figure 22). The MPM2 antibody did not recognize dephosphorylated htau40, but did identify several bands migrating above 98 kDa and a single band migrating around 52 kDa in the bovine brain extract at the completion of the reaction (Figure 22, lanes 1 and 2). An additional MPM2 reactive broad band was present in the brain extract sample containing htau40 (Figure 22, lane 3). To determine the relationship of these MPM2 reactive bands with tau, similar blots were stained for unphosphorylated and phosphorylated tau using the BT2 and AT8 antibodies respectively (Figure 23). The unphosphorylated htau40 (Figure 23, lane 2, Coom,
arrowhead) migrated at 64 kDa and was only recognized by the BT2 antibody. The htau40 increased in apparent molecular weight following in vitro phosphorylation, which is typical of hyperphosphorylated tau (Figure 23, lane 2, Coom, arrow). The phosphorylated htau40 appeared as a broad band ranging from 71-75 kDa, suggesting that multiple subpopulations of phospho-tau were produced from the single htau40 isoform. Both the MPM2 and AT8 phospho-dependent antibodies recognized the in vitro phosphorylated htau40 (Figure 23). In addition, the BT2 antibody also reacted weakly with a portion of the phosphorylated htau40 that migrated at a molecular weight intermediate between that of unphosphorylated htau40 and the AT8 reactive hyperphosphorylated tau. The position of the BT2 reactive band further indicated that subpopulations of phosphorylated tau were formed following incubation with the crude bovine brain extract. The AT8 antibody reacted strongly with the upper portion of the broad phospho-tau band, and was consistent with previous in vitro results using other kinase sources such as a porcine brain extract, cdk2, and cdk5 (Baumann et al., 1993; Drewes et al., 1993). On the other hand, the phosphorylated htau40 recognized by the MPM2 antibody appeared as a doublet. The lower band of the MPM2 doublet co-migrated with the BT2 reactive phospho-tau band, whereas the upper band appeared to co-migrate with the lower portion of the AT8 reactive phospho-tau. These results showed that the MPM2 antibody reacted with htau40 phosphorylated in vitro, and that it recognized a subpopulation of phospho-tau intermediate between dephosphorylated and the AT8 reactive hyperphosphorylated states.
In addition to the phosphorylated tau bands, the MPM2 antibody also stained a single band migrating at a lower molecular weight than unphosphorylated htau40 and several high molecular weight bands (Figures 22 and 23). Based upon the staining with the BT2 and AT8 antibodies, these proteins were present in the bovine brain extract, and were unrelated to the recombinant tau. MPM2 specific phosphorylation of high molecular weight proteins, including microtubule associated proteins that are present in brain extracts, has been reported previously (Tombes et al., 1991).

**Identification of intermediate tau phosphorylation states**

In order to address whether a temporal sequence of phosphorylation occurred that could further distinguish the MPM2 and AT8 reactivity of phosphorylated htau40, a time course of *in vitro* phosphorylation was examined. Recombinant tau was subjected to phosphorylation as in Figure 22, and individual samples were removed for analysis at various time points (Figures 24 and 25). The phosphorylation reaction was carried out in the presence (Figure 24) or absence (Figure 25) of microcystin in order to determine the effect of Ser/Thr protein phosphatases PP1 and PP2A on the immunoreactivity of the phospho-tau epitopes. Previous reports have indicated that both PP2A (Goedert et al., 1992a; Gong et al., 1994b; Merrick et al., 1996) and calcineurin (Gong et al., 1994a) can dephosphorylate the AT8 epitope on tau. As in Figure 22, the endogenous calcineurin was inhibited by the presence of the Ca\(^{2+}\) chelating agent EGTA in the
buffer system. Regardless of the presence of microcystin, htau40 became partially phosphorylated after incubation for 2 hours as indicated by the appearance of a slightly slower migrating band in the coomassie stained gels (Figures 24 and 25, Panel D, lane 2). In both the presence or absence of microcystin, this doublet band was also strongly reactive with the BT2 antibody (Figures 24 and 25, Panel C). However, with continued reaction time, the intensity of the BT2 reactive tau bands decreased in the presence of microcystin (Figure 24, Panel C). At 24 hours (Figure 24, Panel C, lane 9), the BT2 reactive doublet was converted into a single band having reduced immunoreactivity. In the absence of microcystin, the intensity of the BT2 reaction remained elevated, however, the lower band (unphosphorylated tau) decreased in amount upon continued phosphorylation (Figure 25, Panel C). Thus, the BT2 blots and coomassie stained gels revealed that a distinct pattern of intermediate phosphorylated isoforms of tau were progressively formed and further modified during the kinase reaction in the presence or absence of microcystin.

The MPM2 antibody reacted with partially phosphorylated htau40 after 6 hours of reaction in the presence of microcystin (Figure 24, Panel B, lane 4). As the phosphorylation reaction continued, the htau40 became more intensely MPM2 reactive, increased in apparent molecular weight, and shifted from a doublet at 6 hours to a single band at 24 hours. In the absence of microcystin (Figure 25), the MPM2 reactive doublet was not apparent until 8 hours (Panel A, lane 5) indicating that protein phosphatases PP1 and/or PP2A were capable of slowing down the
appearance of the MPM2 phosphoepitope on tau. In comparison with the formation of the MPM2-reactive epitope, the appearance of the PHF phosphoepitope identified by the AT8 antibody was delayed (Figures 24 and 25, Panel B). In addition, formation of the AT8 epitope was extremely sensitive to phosphatase activity in the absence of microcystin (Figure 25). AT8 reactive tau did not appear until 8 hours of incubation when both PP1 and PP2A were inhibited, and the reactivity gradually increased throughout the reaction (Figure 24, Panel B). However, if these phosphatases were not inhibited, the AT8 epitope was not readily phosphorylated (Figure 25, Panel B). Only weak AT8 reactivity was present even after 24 hours of incubation. These results demonstrated that a sequential pattern of tau phosphorylation occurs with the MPM2 epitope being phosphorylated prior to the AT8 epitope. In addition, the MPM2 epitope was much less sensitive to the action of phosphatases. Thus, the MPM2 and AT8 epitopes were biochemically distinct phosphorylation sites.

**Analysis of additional tau phosphoepitopes**

Since the MPM2 and AT8 antibodies did not recognize the same phosphoepitope on tau, three additional tau phosphoepitopes were analyzed using polyclonal antibodies generated against synthetic phosphopeptides (see experimental procedures). All three phosphoserine antibodies, pSer199, pSer396, and pSer262, recognized *in vitro* phosphorylated htau40 within two hours of incubation with a crude fetal (E19) kinase extract (Figures 26 and 27, Panels C-E,
respectively). The intermediate phosphorylated isoforms of tau which migrate as
doublets were identified by the BT2 antibody (Panel F) and coomassie protein stain
(Panel G) as in figures 24 and 25. These phospho-tau doublets were also intensely
stained by the pSer199, pSer396, and pSer262 antibodies (Panels C-E,
respectively). The immunoreactivity of the phosphorylated tau isoforms with these
phosphoserine antibodies gradually increased as the reaction continued, ultimately
producing a broad hyperphosphorylated tau band after 24 hours. The Ser 199, Ser
262, and Ser 396 sites on tau were more rapidly phosphorylated by kinase(s)
present in fetal brain extracts when compared to the MPM2 epitope (Panel A),
which required at least 6 hours of incubation prior to its detection. Furthermore, the
absence of microcystin in the phosphorylation assay (Figure 27) did not affect the
ability of recombinant tau to be phosphorylated on Ser 199, Ser 262, or Ser 396.
These results indicate that these sites are not as sensitive to dephosphorylated by
PP1 and/or PP2A as the AT8 epitope on tau, which was completely lost in the
absence of microcystin (Figure 27, Panel B). Similar results were obtained with the
MPM2, pSer199, pSer262, and pSer396 antibodies in the presence of microcystin
(Figure 26). The rapid appearance of staining with the three phosphoserine
antibodies suggested that the Ser 199, Ser 262, and Ser 396 sites undergo
phosphorylation prior to other tau epitopes such as the MPM2 and AT8 sites. It is
possible that prior phosphorylation on Ser 199, Ser 262, or Ser 396 may be required
for further phosphorylation of tau at other sites including the MPM2 and AT8
epitopes. Finally, the tau phosphorylation pattern indicated that the MPM2 epitope on tau was different from the Ser 199, Ser 202, Thr 205, Ser 262, and Ser 396 sites.

**In vitro phosphorylation of tau by microtubule associated and soluble kinases**

Since the MPM2 antibody recognizes MAP1A, MAP1B, and PHF-tau, we examined whether the kinase(s) that phosphorylate tau on the MPM2 epitope were associated with microtubules and/or different from those kinase(s) that phosphorylate tau on the AT8 site. A series of *in vitro* phosphorylation assays were carried out using fractions obtained from the purification of microtubules as the kinase sources (see experimental procedures). All of the fractions phosphorylated htau40 within a 24 hour time period as shown in the coomassie stained gel (Figure 28, Panel C). The maximal phosphorylation of htau40 occurred using the crude brain supernatant (CBS) and the first warm supernatant lacking microtubules (H₅S) as the kinase sources (Figure 28, lanes 4 and 5 respectively). On the other hand, phosphorylation of htau40 by kinases associated with single (CₛS) or twice cycled (C₂S) microtubule containing fractions, (lanes 6 and 7 respectively) produced a doublet. This tau doublet did not appear to be as highly phosphorylated as the tau in the CBS and HₛS reactions based upon its migration patterns (compare Figure 28, Panel C lanes 6 and 7 to lanes 4 and 5). Interestingly, the AT8 epitope on tau was phosphorylated by kinase(s) present in all fractions (Figure 28, Panel B), with the highest level of reactivity occurring in the CBS (lane 4) and HₛS (lane 5) fractions. The level of AT8 reactivity observed using the CBS or HₛS fractions was
comparable to that of the crude brain extract (lane 3). While kinases present in the microtubule containing fractions phosphorylated htau40 to a lesser extent than the CBS and H,S fractions, these bands were also AT8 reactive. Therefore, phosphorylation of the AT8 epitope on tau was observed with two different groups of kinases, those associated with microtubules and soluble kinases. The htau40 phosphorylated by the CBS and H,S fractions also showed immunoreactivity with the MPM2 antibody at a level similar to that seen using the crude brain extract (Figure 28, Panel A). Interestingly, the C,S and Ç S microtubule containing fractions did not phosphorylate htau40 on the MPM2 epitope. These results indicated that at least one subset of kinase(s) capable of phosphorylating tau on the AT8 epitope were not capable of phosphorylating tau at the MPM2 site. Further, the kinase(s) responsible for the phosphorylation of tau on the MPM2 epitope were not associated with microtubules.

**Comparison of fetal and adult kinases**

Since the presence of the MPM2 phosphoepitope on PHF-tau supports the hypothesis that mitotic or developmental kinases are being reactivated in AD, we compared the *in vitro* phosphorylation of htau40 using a fetal rat brain (E19) kinase extract with kinase extracts isolated from both adult rat and bovine brains (Figure 29). The three kinase extracts, fetal rat (lane 2), adult rat (lane 4), and adult bovine (lane 6), were capable of phosphorylating htau40 (Panel C). In addition, phosphorylated htau40 became both MPM2 (Panel A) and AT8 (Panel B) reactive
in all cases with no obvious difference in the maximal intensity of the reaction between the different extracts. Interestingly, the MPM2 immunoreactive phospho-tau formed with the fetal rat brain extract did not comigrate with the adult extracts (Figure 29, compare lane 2 with lanes 4, 6). Therefore, the overall extent of htau40 phosphorylation in the fetal extract was apparently less as evident by the increased mobility of the MPM2-reactive band. In addition, the time course phosphorylation of htau40 revealed that the MPM2 epitope on tau was phosphorylated within 6 hours using the fetal kinase extract (Figure 27, Panel A) whereas the adult kinase extract (Figure 25, Panel A) took 8 hours to phosphorylate the MPM2 site. Both the change in mobility of the MPM2 reactive phospho-tau and the variation in ability of fetal versus adult kinases to phosphorylate tau is likely due to differences in the presence or absence of specific kinases or their regulation in the fetal and adult brain samples. Regardless, these results indicated that kinases are present in both fetal and adult brains capable of phosphorylating htau40 on both the AT8 and MPM2 epitopes.
DISCUSSION

In the present study we show that recombinant human tau is phosphorylated in vitro on the MPM2 epitope by kinases present in a crude bovine brain extract. Similar extracts have been shown to phosphorylate tau at many of the known PHF phosphorylation sites. These results indicate that PHF-like phosphorylation of tau can be induced by kinases found in normal adult brain tissue. The temporal sequence of tau phosphorylation demonstrates that the MPM2-reactive tau isoform migrates at an intermediate position between fully dephosphorylated recombinant tau and the slowest migrating isoforms of hyperphosphorylated tau detected by the PHF-tau reactive AT8 antibody. MPM2 reactivity is also observed prior to the appearance of the AT8 reactive tau species regardless of whether protein phosphatases PP1 and PP2A were inhibited or not. Thus, in comparison to the AT8 epitope, the MPM2 epitope on tau was more stable to dephosphorylation by phosphatases present in the brain extracts. Both the pattern of phosphorylation and sensitivity to phosphatase clearly indicated that the MPM2 and AT8 phosphoepitopes are distinct, and that phosphorylation of the MPM2 epitope appears to precede phosphorylation of the AT8 epitope. Furthermore, the MPM2 epitope on tau is also clearly distinct from other proline-directed phosphorylation sites at Ser 199 and Ser 396, and a non-proline directed site at Ser 262 located in the microtubule binding domain. These additional sites are readily phosphorylated in comparison to the MPM2 epitope site.
Characterization of several MPM2-reactive peptides and proteins have indicated that many potential MPM2 sites may be proline-directed Ser/Thr phosphorylation sites (Westendorf et al., 1994; Matsumoto-Taniura et al., 1996). Recently, the MPM2 epitope site present on human DNA topoisomerase II was characterized using a series of synthetic phosphopeptides (Ding et al., 1997). A model of the MPM2 phosphoepitope was proposed based upon three criteria: 1) the epitope requires a phosphorylated Ser/Thr with phosphothreonine being preferred, 2) an aromatic amino acid one or two amino acids to the N-terminal side of the phosphothreonine strongly influences recognition, and 3) an aromatic or positively charged amino acid to the C-terminal side of the phosphothreonine further enhances the stability of the complex. This model allows, but does not require, the presence of a proline residue adjacent to the phosphorylated Ser/Thr residue. The model includes both the proline-independent MPM2 sites identified in MAPK (Taagepera et al., 1994) and topoisomerase II (Ding et al., 1997), and most of the proline-directed sites identified previously (Westendorf et al., 1994). Based on these MPM2 epitope criteria, a potential site has been identified in the N-terminal region of MAP1B, and the corresponding MAP1B synthetic phosphopeptide has been shown to react with the MPM2 antibody (Feng and Vandré, manuscript in preparation). In addition, polyclonal antibodies made to the phosphopeptides corresponding to the topoisomerase II site and MAP1B site recognized
phosphorylated htau40 and PHF-tau (Kondratick and Vandré, unpublished observations; Chapter 3). Thus, the MPM2 epitope on tau may share characteristics with other MPM2 reactive sites on topoisomerase II and MAP1B.

PHF-tau has been shown to be phosphorylated at 19-21 Ser/Thr sites (Imahori and Uchida, 1997) including Ser 198, Ser 208, Ser 210, Ser 214, Ser 262, Ser 400, Thr 403, Ser 409, Ser 412, and Ser 413 which are non-proline directed sites, and Ser 199, Ser 202, Thr 212, Thr 217, Thr 231, Ser 235, Ser 396, Ser 404, and Ser 422 which are proline directed. Based upon the existing MPM2 epitope model (Ding et al., 1997), three potential MPM2 sites have been identified in the tau sequence. One of these potential sites, Thr 377, fits the model by having aromatic amino acids on both the N- and C-terminal sides. This site can be eliminated, however, since there is no biochemical evidence to indicate that this site is phosphorylated in vivo or in vitro. The other two potential MPM2 sites on tau contain an aromatic amino acid to the N-terminal side of a phosphorylated serine. One of these sites may be related to the proline-directed PHF-1 epitope, Ser 396/404. The other site contains both non-proline directed, and proline directed residues at Ser 198 and Ser 199. Each of these potential MPM2 epitope sites are phosphorylated in AD and found on PHF-tau. Using antibodies selective for phosphorylated Ser 396 and Ser 199 a phosphorylation pattern that was distinct from that seen with the MPM2 antibody was observed. This result implies that phosphorylation of tau on either Ser 396 or Ser 199 alone did not confer MPM2
reactivity. Moreover, phosphoserines appear to be less reactive with the MPM2 antibody than phosphothreonines implying that both of these potential MPM2 sites may be weak recognition sites. It is also possible that phosphorylation of residues adjacent to Ser 396 or Ser 199 may modulate antibody recognition. Recently, two other PHF-tau antibodies AT10 and PHF-27 were shown to recognize dual phosphorylation sites (Hoffman et al., 1997). The AT10 antibody requires the phosphorylation of both a proline-directed site, Thr 212, and a proline independent site, Ser 214, for recognition. Both AT10 and PHF-27 are specific for PHF-tau and do not react with fetal tau, normal adult biopsy-derived tau, or biopsy-derived tau (Hoffmann et al., 1997). Thus, it appears that PHF specific antibodies recognize multiple phosphorylated residues on tau as opposed to individual phosphorylated residues. Our previous results indicate that the MPM2 antibody also recognizes an epitope specific to PHF-tau, since fetal and adult rat tau were not reactive (Kondratick and Vandré, 1997). Therefore, the MPM2 antibody may be another member of a class of “true” PHF specific antibodies like AT10 and PHF-27.

Several cell cycle regulated kinases including MEK (Taagepera et al., 1994), MAPK (Kuang and Ashom, 1993), and cdc2 (Westendorf et al., 1994) kinases have been shown to phosphorylate MPM2 epitopes on selected target proteins. Since the MPM2 antibody recognizes a phosphoepitope associated with mitotic cells, it is possible that kinases present in the brain extract that phosphorylate the MPM2 epitope site on tau may be related to kinases involved in cell-cycle regulation. In the
present study, the kinases responsible for the phosphorylation of the MPM2 and
AT8 epitopes on tau appear to be distinct. While soluble kinases were capable of
phosphorylating tau at both of these sites, the microtubule-associated kinases were
only able to phosphorylate tau on the AT8 epitope. Also, while fetal (E19) and adult
brain extracts were both capable of phosphorylating tau on the MPM2 and AT8
epitopes, phosphorylation of the MPM2 epitope occurred earlier using the fetal brain
extract. These results indicate a potential difference in the kinase activities present
in early stages of brain development versus those found in the adult brain.

Numerous kinases are capable of phosphorylating tau in vitro including:
protein kinase A and C (Lituresky and Johnson, 1992; Correas et al., 1992),
Ca²⁺/calmodulin kinase II (Baudier and Cole, 1987), casein kinase II (Steiner et al.,
1990), glycogen synthase kinase-3 (GSK-3) (Hanger et al., 1992; Mandelkow et al.,
1992), mitogen-activated protein kinase (MAPK) (Drewes et al., 1992; Goedert et
al., 1992a), p110<sup>mark</sup> (Drewes et al., 1995), p34<sup>cdc2</sup> kinase (Ledesma et al., 1992;
Vulliet et al., 1992), and cdc2-like kinases (Baumann et al., 1993). Several of these
kinases are proline-directed kinases, including the MAPK family, GSK-3α and β,
and cdk5, which phosphorylate tau on Ser/Thr-Pro sites. All three of these kinases
are found in neurons and copurify with microtubules (Baumann et al., 1993). Both
cdk5 and cdc2 kinases can phosphorylate tau on Ser 202 and Thr 205 which
confers AT8 reactivity (Goedert et al., 1995; Billingsley and Kincaid, 1997). Thus,
cell cycle dependent kinases may in part be responsible for the phosphorylation of
this and other epitopes in AD including the MPM2 epitope. In addition, other kinases such as the MAPK family and GSK-3α can phosphorylate the Ser 202 site indicating that multiple kinases working in conjunction can produce hyperphosphorylation of tau and PHF phosphoepitopes. Cooperative phosphorylation of tau has been seen with the cdk5 and GSK-3β kinases (Ishiguro et al., 1995). In these studies, GSK3-β was shown to have enhanced activity after prior phosphorylation of tau with cdk5. GSK3-β can phosphorylate tau on Ser 199, Thr 231, Ser 396, and Ser 413 in the presence of cdk5, and produce a shift in apparent molecular weight (Imahori and Uchida, 1997). Furthermore, GSK3-β could phosphorylate three non-proline directed sites, Ser 198, Ser 400, and Ser 409, if tau was first phosphorylated on Ser 202, Ser 404, and Ser 413 (Imahori and Uchida, 1997). Although we show that the MPM2 epitope on tau was not phosphorylated by microtubule-associated kinases alone, it is possible that prior phosphorylation of tau by these kinases may enhance phosphorylation at the MPM2 epitope by soluble kinases. Thus, formation of the MPM2 epitope on tau may require the cooperation between proline directed kinases associated with MT such as cdk5 and GSK-3β, and an unidentified non-proline directed kinase related to the MPM2 epitope kinases capable of phosphorylating topoisomerase II and MAPK.

Hyperphosphorylated PHF-tau is probably not a homogenous population, but rather a mixture of distinct phosphoisoforms of tau. Recently, two dimensional gel electrophoresis was utilized to show that PHF-tau is indeed heterogeneously
phosphorylated (Sergeant et al., 1997). The generation of a heterogeneous population of tau phosphoisoforms has also been shown in vitro using a single kinase, ERK2 (Roder et al., 1997). The ERK2 kinase was shown to phosphorylate up to 13 sites on tau to varying degrees, many concurrently. These results suggest that several distinct phosphoisoforms of tau could coexist having both the same shift in apparent molecular weight and similar immunoreactivity with selected phosphorylation dependent antibodies. In addition, some species of phosphorylated tau were not detected by phosphoepitope specific antibodies despite incorporating phosphate at the corresponding amino acid in the epitope site. These results imply that multiple alternative phosphorylation states influence antibody recognition of tau. For example, the MPM2 epitope is generated prior to the formation of the AT8 epitope. However, MPM2 does not appear to recognize all of the AT8 reactive hyperphosphorylated tau. Thus, subsequent phosphorylation of the AT8 epitope may influence the binding of the MPM2 antibody.

The in vitro phosphorylation of the MPM2 epitope on tau indicates that kinases related to those involved in mitotic regulation and/or fetal development are also present in the adult brain. However, since adult neurons are clearly postmitotic, this implies that these kinases are highly regulated and have discrete functions in the adult as compared to the fetal brain. Pyramidal neurons from the hippocampus of AD patients containing NFTs were shown to have elevated levels of active cdc2 kinase, and this kinase was also shown to be associated with PHF-
tau (Vincent et al., 1997). Furthermore, Vincent and colleagues hypothesize that upstream regulators of cdc2 kinase such as wee-1 and cdc25, which are known MPM2-reactive phosphoproteins, are the candidate MPM2 antigens localized in degenerating AD pyramidal neurons. However, we have shown that isolated PHF-tau from AD brains is MPM2 reactive (Kondratick and Vandré, 1996; Chapter 1), and further demonstrate in this study that recombinant human tau can be phosphorylated \textit{in vitro} on the MPM2 epitope. Since hyperphosphorylated tau is the major protein associated with NFTs in degenerating AD neurons, the candidate MPM2 antigen in these cells is most likely PHF-tau. It is possible, however, that other MPM2-reactive proteins such as wee-1 and cdc25 contribute to the MPM2 staining seen in AD brain sections, but this remains to be established.

Deregulation of mitotic and/or fetal kinases, perhaps through reactivation of fetal-like conditions, may partially account for the hyperphosphorylation of tau and associated degeneration of neurons, loss of synaptic connections, and development of dementia common to AD. Determination of the regulatory components involved in the altered phosphorylation pathways in AD neurons will lead to a better understanding of the molecular mechanisms underlying Alzheimer's disease and other neurodegenerative disorders. Examination of a potential role for the MPM2 mitosis-associated epitope in this process will require that the site on tau be defined biochemically; and we are actively pursuing that objective using a series of both deletion and site-directed mutants of tau.
Figure 22. MPM2 reactivity of phosphorylated tau.

Htau40 was phosphorylated for 0 hr (lane 1) and 24 hr (lane 3) by kinases present in the crude bovine brain extract. In addition, the crude kinase extract was incubated for 24 hours without tau present (lane 2) as a control for phosphorylation of endogenous proteins. The phosphorylation reaction was carried out as described in the experimental procedures with 1 µM microcystin present in the reaction buffer. The arrow indicates the position of phosphorylated htau40. The molecular weight markers from top to bottom are as follows: 98, 64, 50, 36, and 30 kDa.
Figure 23. Immunoblot analysis of *in vitro* phosphorylation of htau40.

Both unphosphorylated (lane 1, arrowhead) and phosphorylated (lane 2, arrow) htau40 were analyzed for immunoreactivity with the MPM2, AT8, and BT2 antibodies. In addition, a coomassie stained gel (COOM) was used to show the protein composition of each sample. The phosphorylation reaction was carried out as described in the experimental procedures for 24 hours with 1 μM okadaic acid substituted for microcystin. The multiple lower molecular weight bands seen in the coomassie stained gel were determined to be proteins present in the kinase extract (data not shown). The molecular weight markers from top to bottom are as follows: 98, 64, 50, 36, and 30 kDa.
FIGURE 23
Figure 24. Time course phosphorylation of htau40 in the presence of microcystin.

Recombinant htau40 was phosphorylated using a crude brain extract containing 1 μM microcystin as described in the experimental procedures. Samples were removed and the reaction stopped at 0, 2, 4, 6, 8, 10, 12, 18, and 24 hrs. All of the time points were analyzed for immunoreactivity with the MPM2 (Panel A), AT8 (Panel B), and BT2 (Panel C) antibodies. In addition, the time points were also stained with coomassie blue (Panel D) to examine the mobility of the different subpopulations of phosphorylated htau40.
FIGURE 24
Figure 25. Time course phosphorylation of htau40 in the absence of microcystin.

The phosphorylation of htau40 was carried out as in Figure 23 except microcystin was not present in the reaction mixture. The time points examined were 0, 2, 4, 6, 8, 10, 12, 18 hr, and 24 hrs. All of the time points were analyzed with the MPM2 antibody (Panel A), AT8 antibody (Panel B), BT2 antibody (Panel C), and coomassie blue protein stain (Panel D).
FIGURE 25
Figure 26. Temporal sequence of tau phosphoepitope formation in the presence of microcystin.

The phosphorylation reaction was carried out as in Figure 24 except a crude fetal (E19) brain extract was used as the kinase source. The time points examined were 0, 2, 4, 6, 8, 10, 12, 18, and 24 hrs. All the time points were analyzed with the MPM2 (Panel A), AT8 (Panel B), pSer199 (Panel C), pSer396 (Panel D), pSer262 (Panel E), and BT2 (Panel F) antibodies and coomassie blue protein stain (Panel G).
Figure 27. Temporal sequence of tau phosphoepitope formation in the absence of microcystin.

The phosphorylation of htau40 was carried out as in Figure 25 except a crude fetal rat brain (E19) extract was used as the kinase source. The time points examined were 0, 2, 4, 6, 8, 10, 12, 18, and 24 hrs. All of the time points were analyzed for immunoreactivity with the MPM2 (Panel A), AT8 (Panel B), pSer199 (Panel C), pSer396 (Panel D), pSer262 (Panel E), and BT2 (Panel F) antibodies. In addition, each time point was also stained with coomassie blue (Panel G) to examine the mobility of the different subpopulations of phosphorylated htau40. The phosphoserine antibodies were raised against corresponding synthetic peptides containing a single phosphorylated serine residue (see experimental procedures).
Figure 28. *In vitro* phosphorylation of htau40 using fractions from microtubule purification.

Recombinant htau40 was phosphorylated for 24 hours in the presence of microcystin using different fractions obtained from the isolation of microtubules as the kinase sources (see experimental procedures). Immunoblots were analyzed using the MPM2 (Panel A) and AT8 (Panel B) antibodies as well as coomassie blue protein stain of the samples (Panel C). The kinase fractions used were as follows: crude bovine brain extract (lanes 2 and 3), crude brain supernatant, CBS (lane 4), microtubule depleted supernatant, H1S (lane 5), single cycled microtubules, C1S (lane 6), and twice cycled microtubules, C2S (lane 7). The two control samples are: htau40 only control (lane 1) and an immediately killed reaction or 0 hr incubation (lane 2).
Figure 29. Phosphorylation of htau40 using fetal and adult brain kinase extracts.

Unphosphorylated (odd lanes) and phosphorylated (even lanes) htau40 was analyzed with MPM2 (Panel A) and AT8 (Panel B) antibodies in addition to a coomassie blue stained gel (Panel C). Htau40 was phosphorylated using kinase extracts isolated from fetal rat (lanes 1 and 2), adult rat (lanes 3 and 4), and adult bovine (lane 5 and 6) brains.
CHAPTER 3

Localization of the Mitosis-Associated MPM2 Phosphoepitope on the Neuronal Microtubule Associated Protein Tau

INTRODUCTION

The phosphorylation of proteins on Ser/Thr and Tyr residues is a mechanism by which a cell can regulate various protein functions, protein-protein interactions, and cellular processes such as mitosis. During mitosis or M-phase of the cell cycle, the level of protein phosphorylation dramatically increases as a result of the activation of multiple protein kinases. These mitotic kinases target specific families of mitotic phosphoproteins, which include structural proteins and other mitotic regulators. The major kinase responsible for the progression of cells into mitosis is the cdc2-cyclin B complex or cdc2 kinase. The cdc2 kinase is a proline-directed kinase phosphorylating Ser/Thr-Pro-X-(Lys/Arg) sites on proteins. Interestingly, the activity of the cdc2 kinase is regulated by phosphorylation of its Thr 14 and Tyr 15 residues by the wee1 and myt1 kinases, which renders the protein inactive. Active cdc2 kinase is formed at the onset of mitosis, or G\textsubscript{2}/M boundary, by dephosphorylation of both regulatory sites by Cdc25, a dual-specificity
phosphatase. Furthermore, Cdc25 is regulated by phosphorylation in its N-terminal regulatory domain at mitosis and this domain can be phosphorylated by Plx1 kinase (Kumagai and Dunphy, 1996). Thus, multiple phosphorylation cascades with numerous players are involved in the progression of a cell through mitosis.

A powerful tool for studying these phosphorylation cascades are phospho-specific antibodies. One of these antibodies, the MPM2 antibody reacts with a set of 40 or more phosphoproteins (Davis et al., 1983), which are synthesized during interphase and become phosphorylated when the cell enters mitosis (Kuang et al., 1989). The MPM2 antibody is a monoclonal antibody raised against a mitotic HeLa cell extract which preferentially stains mitotic cells of different species by indirect immunofluorescence (Davis et al., 1983). The mitotic structures reactive with the MPM2 antibody are the kinetochores, centrosomes, chromosomes, kinetochore fibers, and midbodies; all important components of the mitotic machinery (Vandré et al., 1984; Vandré et al., 1991). Furthermore, microinjection of the MPM2 antibody into *Xenopus* oocytes inhibits oocyte maturation induced by progesterone, and immunodepletes cdc2 kinase activity from both mitotic HeLa cells and mature oocyte extracts (Kuang et al., 1989). Thus, the addition of the MPM2 antibody to these cells blocks the onset and completion of mitosis. Additional evidence obtained from *Xenopus* egg extracts reveals that MPM2 inhibition of the completion of mitosis is due in part to the interaction of the antibody with components of the cyclosome or anaphase promoting factor (King et al., 1995). The cyclosome is
responsible for the destruction of cyclin B and subsequent exit of the cell from
mitosis; thus, binding of the MPM2 antibody to this complex inhibits its normal
function. Another in vitro study shows that the MPM2 antibody can block the
nucleation of MTs from mitotic centrosomes indicating that MT nucleation and
subsequent formation of the mitotic apparatus is modulated by phosphorylation of
centrosomal proteins containing the MPM2 epitope (Centonze and Borisy, 1990).
Several proteins are recognized by the MPM2 antibody including MAP1 and MAP4
(Vandré et al., 1986; Vandré et al., 1991), wee1 (Mueller et al., 1995a), myt1
(Mueller et al., 1995b), Cdc25 (Kuang et al., 1994), ERK2 (Taagepera et al., 1994),
topoisoromerase II (Taagepera et al., 1993), NIMA (Ye et al., 1995), casein kinase II
and several other MPM2-reactive phosphoproteins or MPPs of unknown function
(Matsumoto-Taniura et al., 1996).

The variety of phosphoproteins recognized by the MPM2 antibody suggests
that a common phosphorylation site is present within these proteins. Several
studies defining the MPM2 epitope have been published in recent years. Most
reports suggest that a phosphothreonine residue is recognized by the antibody
along with surrounding amino acids that influence antibody binding (see Figure 33
for reactive sequences ) (Taagepera et al., 1994; Westendorf et al., 1994; Ding et
al., 1997; Yaffe et al., 1997). A series of experiments using a pentadecapeptide
expression library phosphorylated by M-phase kinases indicated that the MPM2
epitope sequence consists of a string of five amino acids with the phospho-Thr/Ser
residue followed by a proline residue (Westendorf et al., 1994). The most common sequence found to be MPM2 reactive was LTPLK, although FTPLQ was also MPM2 reactive and exhibited a higher antibody binding affinity. Interestingly, the KTPVK sequence was not recognized by the MPM2 antibody suggesting that a positively charged amino acid N-terminal to the phosphothreonine inhibits the binding of the antibody; however, a positively charged amino acid C-terminal to the phosphorylated residue does not affect antibody recognition. Thus, Westendorf et al. (1994) conclude that the MPM2 epitope required the following recognition sequence: a hydrophobic residue-Thr/Ser-Pro-hydrophobic or basic amino acid.

Identification of an MPM2 epitope on activated ERK2, however, did not fit this epitope model (Taagepera et al., 1994). These results imply that Westendorf et al. (1994) may have only defined a subset of MPM2 epitopes. The ERK2 sequence determined to be MPM2 reactive is not a proline-directed site, but rather a phospho-Thr-Glu-Tyr sequence. The phosphorylation of both the Thr and Tyr at this site are known to be required for full kinase activity of the ERK2. Phosphorylation of the Tyr residue was not required for MPM2 reactivity, but amino acid substitutions at this position affected the interaction between the MPM2 antibody and ERK2. A Tyr-to-Phe substitution resulted in strong MPM2 reactivity, whereas a Tyr-to-Glu change resulted in reduced reactivity below the level seen with the wild-type protein. In addition, substitution of the Thr residue with an Ala eliminated MPM2 binding. These results demonstrate that a negatively charged amino acid C-terminal to the
phosphorylated residue and a proline-directed phosphorylation site are not necessary for MPM2 reactivity. This study also suggested that an aromatic amino acid C-terminal to the phosphorylated residue may influence antibody binding.

Based on this limited analysis of the MPM2 epitope, Ding et al. (1997) identified and partially characterized a putative MPM2 site on topoisomerase II using a series of synthetic phosphopeptides. A revised MPM2 epitope model was derived from these results (Figure 36). Briefly, the three criteria for the MPM2 epitope are: 1) a phosphorylated residue preferably threonine, 2) an aromatic amino acid N-terminal to the phosphorylated residue, and 3) an aromatic or positively charged amino acid C-terminal to the phosphorylated residue. This study also states that maximal MPM2-substrate affinity is seen when all three criteria are met, and that native MPM2 reactive proteins will contain this sequence arrangement. Interestingly, this epitope model did not require that the phosphorylation site be proline-directed, but allows for this possibility and incorporates the previously described MPM2-reactive sequences reported by Westendorf et al. (1994). Further analysis of the MPM2 epitope on topoisomerase II was carried out using a polyclonal antibody, PTE1, which was generated against the phosphorylated topoisomerase II peptide 2 (Figure 33) (Ding, 1996). The PTE1 antibody recognizes a set of phosphoproteins from mitotic HeLa cell lysates and components of the mitotic apparatus, including chromosomes, spindle poles, and the midbody (Ding et al., unpublished data). These results were similar to those obtained with the
MPM2 antibody indicating that the PTE1 and MPM2 epitopes are very similar. In a related study, a potential MPM2 epitope on MAP1B was identified (Figure 33), and a polyclonal antibody to the corresponding phosphopeptide was generated and designated PMB1 (Feng, 1997). The PMB1 antibody also stains the spindle poles of cells in early anaphase indicating that the PMB1 is a MPM2-like antibody.

The previous two chapters demonstrated that the MPM2 antibody recognizes PHF-tau and \textit{in vitro} phosphorylated recombinant full-length human tau. The objective of this chapter is to address the identification of the MPM2 epitope on tau. Different tau isoforms and deletion mutants were phosphorylated \textit{in vitro} using the bovine brain extract described in Chapter 2, and analyzed for their ability to react with the MPM2 antibody on immunoblots. Based on the epitope model proposed by Ding et al. (1997), two potential MPM2 sequences were identified in tau. These two sites were evaluated for their ability to interact with the MPM2 antibody following phosphorylation of deletion mutants for the regions containing the speculated MPM2 sequences. The results from these experiments has narrowed down the region on tau that contains the MPM2 epitope allowing for future identification by site-directed mutagenesis.
Antibodies

Several antibodies were used to identify phosphoepitopes present on tau including AT8, MPM2, pSer199, pSer262, pSer396, PTE1, and PMB1. The AT8 monoclonal antibody (Biosource International, Camarillo, CA, U.S.A.) was raised against PHFs and recognizes tau when it is phosphorylated on both Ser 202 and Thr 205 sites. Both of these sites are found phosphorylated on native fetal tau and PHF-tau. The mitosis-associated MPM2 monoclonal antibody (Upstate Biotechnology International, Lake Placid, NY, U.S.A.), which was raised against a mitotic HeLa cell extract, recognizes a series of phosphorylated proteins including MAPs (Vandré et al., 1991), PHF-tau (Kondratick and Vandré, 1996), and in vitro phosphorylated recombinant human tau (Kondratick et al., submitted manuscript; Chapter 2). In addition, synthetic phosphopeptides were utilized to produce polyclonal antibodies to tau residues pSer199, pSer262, pSer396 (Kondratick et al., submitted manuscript; Chapter 2). The phosphorylation specific PTE1 antibody that recognizes topoisomerase II, and the PMB1 antibody that recognizes MAP1B, were also used (Ding et al., manuscript in preparation). Three additional antibodies were used to identify tau, BT2, N-term, and C-term. The BT2 antibody (Biosource International) reacts with the amino acid sequence RSGYS between positions 194-198 and will not recognize tau when phosphorylated in this region. Both N-term
and C-term antibodies recognize tau independent of phosphorylation state and were generated against synthetic peptides, MAEPRQFEFVMDHAC-amide and VDSPQLATLAD, respectively, by Sushovita Mukherjee in this lab.

**Preparation and Transformation of Competent Cells**

*E. coli* BL21(DE3) cells were a gift from Smita Patel (Ohio State University, Columbus, OH) and were made competent by the following procedure. An overnight culture of BL21(DE3) cells, 1 ml, was used to inoculate 10 ml of sterile LB medium, which was then incubated at 37°C for growth until an OD₆₀₀ of 0.2-0.4 was reached. After reaching the appropriate density, the cells were poured into prechilled polypropylene tubes and placed on ice for 10 minutes. The cells were pelleted by centrifugation at 750-1000 g for 10-15 min at 4°C and the resulting supernatant was removed. The cell pellet was resuspended by gentle pipetting in 4 ml (approximately 1/3 volume of the original culture suspension) prechilled and filter-sterilized RF1 buffer (100 mM RbCl, 50 mM MnCl₂, 30 mM potassium acetate, 30 mM CaCl₂, and 15% (w/v) glycerol, pH 5.8 with acetic acid). The resuspended cells were incubated on ice for 1 hr, pelleted at 4°C, and the supernatant was discarded prior to resuspension in 1 ml (approximately 1/12.5 of original volume) sterile and cold RF2 buffer (10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂, and 15% (w/v) glycerol, pH 6.8 with NaOH). The resuspended cells were incubated on ice for an additional 30 minutes. The competent cells were distributed into 1.5 ml sterile microfuge tubes in 200 µl aliquots, frozen with liquid N₂, and stored at -70°C.
One tube of BL21(DE3) competent cells was thawed on ice and inoculated with 200-500 ng (1 µl) plasmid DNA containing tau deletion mutants (Carmel et al., 1996), htau24, or htau34 (Goedert and Jakes, 1990) sequences under control of the T7 RNA polymerase promoter (McLeod et al., 1987; Studier et al., 1990). The cells plus plasmid were incubated on ice for an additional 30 minutes followed by heat shock at 42°C for 1 minute and immediately chilled on ice for 2 minutes allowing the bacteria to take up the plasmid DNA. The transformed cells were allowed to recover and grow for 1 hr at 37°C with gentle shaking after the addition of 800 µl of sterile LB. Approximately, 200 µl of transformed cells were spread on LA plates containing 100 µg/ml ampicillin and incubated overnight at 37°C.

Single colonies of transformed cells were selected for expansion in 5 ml sterile LB plus ampicillin followed by induction of tau protein by the addition of IPTG to a final concentration of 0.5 mM after cells had reached an OD_{600} ~0.6. Both uninduced and induced cell pellets were lysed and resuspended in 1% hot SDS followed by sonication to break-up the DNA. Protein concentrations were determined by BCA assay (Pierce) and 20 µg of each sample was analyzed by 10% SDS-PAGE, subsequent coomassie blue stain, and in some cases immunoblot analysis with anti-tau antibodies. Colonies that showed induction of tau proteins were used for large scale purification and phosphorylation assays.
Tau deletion mutant purification

Plasmid DNA for tau deletion mutants, Δ162-209 and Δ321-441 were a gift from Jeff Kuret (Northwestern University, Chicago, IL). Each tau mutant was transformed into BL21(DE3) competent cells for expression of the mutant protein and purified by a modified procedure based on previous published protocols (Carmel et al., 1996; Carmel et al. 1994; Goedert and Jakes, 1990; Sambrook et al., 1989). The cells were grown to an \( \text{OD}_{600} \) of approximately 0.6 prior to induction with IPTG at a final concentration of 0.5 mM for 2-2.5 hours. The induced cells were pelleted by centrifugation at 6000 g for 15-20 minutes at 4°C followed by removal of the supernatant and freezing of the pellet in liquid nitrogen. The frozen pellets were resuspended by gentle pipetting in approximately 1:1 (w/v) cold lysis buffer (20 mM Tris pH 8.0 containing 500 mM NaCl, 1 mM DTT, 100 μM EDTA, 100 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A, 0.2 mg/ml lysozyme, and 10 μg/ml DNase I). The cells were transferred to a polypropylene tube and frozen with liquid nitrogen. The frozen cells were allowed to thaw in a 37°C water bath followed by three more cycles of freeze/thaw to lyse the cells. After the last cycle, the lysed cells were subjected to high-speed centrifugation at 50,000 rpm for 1 hour at 4°C to pellet the cellular debris. The high-speed supernatant was removed and boiled for 10 minutes to remove all heat labile proteins. After the boiled supernatant had been chilled on ice for 10 minutes, a supernatant containing the heat stable proteins was obtained by centrifugation at 20,400 g for 15 minutes at 4°C. The enriched tau
supernatant was dialyzed overnight into the PIPES buffer used in the htau40 purification procedure and further purified using FPLC according to the htau40 protocol (Chapter 2).

**Purification of htau24 and htau34**

Htau24 and htau34 plasmid DNA were gifts from Michel Goedert (MRC Laboratory of Molecular Biology, Cambridge, England). Both tau isoforms were transformed into BL21(DE3) as described above and purified according to the htau40 purification protocol (Chapter 2).
RESULTS

MPM2 reactivity of htau24 and htau34

It has been suggested that the presence of the N-terminal inserts in the different isoforms of tau may provide important structural determinants that can modulate the specificity of different kinases for tau (Singh et al., 1996). This would potentially lead to the formation of tau isoforms having different phosphorylation states due to variations in the sites phosphorylated on each isoform. Since PHF-tau and in vitro phosphorylated full-length recombinant tau are MPM2 reactive proteins, whereas fetal and adult rat tau are not recognized by this antibody, it was possible that not all tau isoforms contain the MPM2 epitope and/or are capable of being phosphorylated on this site. To address this question, and the possibility that the MPM2 site was located within the N-terminal inserts, two additional recombinant human tau isoforms were examined and compared to htau40 using the in vitro phosphorylation assay.

The two smaller tau isoforms differ from htau40 by either containing zero (htau24), or one (htau34), N-terminal insert; however, all three tau isoforms retain an equal number of MT binding repeats (Figure 30). Both tau isoforms were expressed under the control of the T7 RNA polymerase promotor (McLeod et al., 1987; Studier et al., 1990) and purified in the same manner as htau40 (Figure 31, panel A). Htau24 (arrow) and htau34 (arrowhead) were clearly induced within 2
hours of adding IPTG (compare lane 1 with 2 for htau34 and lane 4 with 5 for htau24), and became enriched after removal of the heat-labile proteins after boiling of the lysed cell extracts (lanes 3 and 6). The enriched heated supematant fractions were further purified by ion-exchange chromatography yielding primarily a single protein band with some minor degradation products (Figure 31, panel B). Interestingly, the htau24 and htau34 proteins migrated at apparent molecular weights of 52 and 59 kDa which was higher than their predicted molecular weights of 40 and 43 kDa, respectively (Figure 31, panel B). The htau40 isoform also showed a similar variation in migration on SDS-PAGE, which has been noted in the preceding chapter. These results were consistent with published reports showing that all tau isoforms, both the recombinant and native isoforms, run abnormally on SDS-PAGE (Goedert and Jakes, 1990).

The purified tau isoforms were subjected to phosphorylation using the bovine brain extract under the experimental conditions established for htau40 in Chapter 2 (Figure 32). Both htau24 (lanes 1 and 2) and htau34 (lanes 3 and 4) were phosphorylated by the kinases present in the bovine brain extract and shifted in apparent molecular weight, which is characteristic of many phosphorylated proteins including htau40 (panel A). The two smaller isoforms of tau appeared as broad bands after phosphorylation, with htau24 migrating as a diffuse doublet. These results suggested that subpopulations of differentially phosphorylated forms of htau24 and htau34 exist, which has also been shown with the phosphorylated
The ability of htau24 and htau34 to be phosphorylated on the same sites as htau40 was examined by reactivity of the various isoforms with the AT8 (panel B), MPM2 (panel C), and BT2 (panel D) antibodies. The BT2 antibody, which recognizes a dephosphorylated epitope on tau, reacted strongly with the unphosphorylated forms of htau24, htau34, and htau40. In addition, this antibody also recognized a portion of the phosphorylated tau that was not fully phosphorylated or phosphorylated in the region containing the BT2 epitope. This result was consistent with that previously demonstrated for htau40 as shown in Chapter 2. Since the region containing the BT2 epitope is common to all three isoforms tested, it was not surprising that a subset of htau24 and htau34 also reacted with the BT2 antibody after phosphorylation. The phosphorylated htau24 recognized by BT2 migrates as doublet, differing from the other two isoforms that migrated as single bands. This difference in migration pattern of the phosphorylated tau isoforms suggested a variation in the ability of htau24 to be phosphorylated by certain kinases present in the brain extract; however, additional studies are necessary to determine if this is indeed the case. The two phospho-dependent antibodies, AT8 and MPM2, recognized all three recombinant isoforms of tau after phosphorylation (panels B and C, respectively). The level of reactivity of each isoform is equivalent for both the antibodies. Thus, these results indicated that the presence of the N-terminal inserts does not affect
the ability of tau to be phosphorylated on the MPM2 epitope. Furthermore, these results also demonstrated that the MPM2 epitope was not located within amino acids 45-102 (numbering according to the longest isoform of tau).

Reactivity of tau with MPM2-like antibodies

To determine if the MPM2 epitope on tau shared the same characteristics as previously described epitopes on topoisomerase II (Ding et al., 1997) and MAP1B (Feng, 1997), PHF-tau and in vitro phosphorylated htau40 were analyzed for reactivity with polyclonal antibodies PTE1 and PMB1 raised against the potential MPM2 sequences determined in these two proteins, respectively. Both polyclonal antibodies were generated against synthetic phosphopeptides containing a similar sequence of amino acids (Figure 33). The potential MPM2 site on topoisomerase II was determined through analysis of published MPM2 reactive sequences (Figure 33) and further characterized by amino acid substitutions (Ding et al., 1997). Based on the proposed MPM2 epitope model (Ding et al., 1997) (Figure 36), the MAP1B site was determined and the PMB1 antibody generated. Both PTE1 and PMB1 antibodies were shown to react with mitotic phosphoproteins, and to be MPM2-like antibodies (Ding, 1996; Feng, 1997).

PHF-tau was isolated from an AD brain according to the procedure described in Chapter 1 and tested for its ability to interact with the MPM2-like antibodies (Figure 34). Approximately 40 µg of PHF-tau was separated on 10% SDS-PAGE
followed by immunoblot analysis with control antibodies AT8 (lane 1), BT2 (lane 2), and MPM2 (lane 3). As expected, both AT8 and MPM2 reacted with the PHF-tau triplet or A68 bands, whereas BT2 did not recognize PHF-tau. Both MPM2-like antibodies, PTE1 (lane 4) and PMB1 (lane 5) also recognize PHF-tau; however, the PTE1 antibody only reacted with the upper two A68 bands having apparent molecular weights of 68 and 64 kDa. This preference in staining of the 68 and 64 kDa bands over the 60 kDa band has been observed previously with the MPM2 antibody (Chapter 1, Figure 19). These results suggest that the phosphorylated forms of tau making up the 60 kDa band may be phosphorylated to a lesser extent on the MPM2 epitope. The level of phosphorylation may also vary between individuals, since some PHF-tau samples show stronger MPM2 reactivity of the 60 kDa band (Chapter 1, Figure 20). Nonetheless, the PTE1 and PMB1 antibodies recognize PHF-tau in a similar manner as the MPM2 antibody.

In addition to PHF-tau, in vitro phosphorylated htau40 was examined for reactivity with the PTE1 and PMB1 antibodies (Figure 35). MPM2, AT8, and BT2 all reacted with the phosphorylated htau40 (lane 2) as excepted and as shown previously in Chapter 2. The PTE1 antibody only recognized the htau40 after phosphorylation, and this band appeared to comigrate with the MPM2-reactive htau40 species. Furthermore, the PTE1-reactive htau40 seemed to recognize the lower portion of the AT8 reactive band, which was also the case for the MPM2 antibody. This result suggested that the PTE1 and MPM2 antibodies may be
recognizing similarity phosphorylated subpopulations of htau40 and possibly the same tau epitope. In comparison, the PMB1 antibody recognized both dephosphorylated (lane 1) and phosphorylated htau40. The reaction of the PMB1 antibody with the unphosphorylated htau40 was shown to be due to non-specific reactivity of the crude antiserum, and could be eliminated with the incorporation of Tween 20 in the immunoblot buffers (Feng and Vandré, unpublished observations). The reactivity of the phosphorylated htau40 with the PMB1 antibody revealed a broad band that comigrated with the AT8 reactive species that was also recognized by PMB1 in the presence of Tween 20 containing buffers (data not presented). Taken together, these results implied that the MPM2 epitope on tau may contain similar attributes to the identified sites on topoisomerase II and MAP1B.

**Determination and analysis of potential MPM2 sites on tau**

The MPM2 epitope model proposed by Ding et al. (1997) was used to determine potential MPM2 sites on tau (Figure 36). Three possible sites were identified that fit the model. The Thr 377 site was eliminated from consideration, since this site has never been shown to be phosphorylated either biochemically or by immunological methods in any other study. The two remaining sites were the Ser 396 residue within the C-terminal targeting domain, and the Ser 198/Ser 199 dual phosphorylation site within the proline-rich region N-terminal to the MT binding repeats. Both potential sites contained a phosphorylated serine residue and were preceded by an aromatic amino acid, tyrosine, in the +1 or +2 position. It is
important to point out that the Ser 396 and Ser 199 sites are proline-directed, while the Ser 198 is not followed by a proline residue. Furthermore, the phosphorylation of adjacent serine residues may alter antibody binding to the epitope by either enhancing or diminishing antibody-epitope interaction.

A series of experiments to delineate the MPM2 epitope on tau involved the phosphorylation of two deletion mutants for the regions of tau containing the potential MPM2 sites (Figure 37). The N-terminal deletion mutant had amino acids 162-209 removed, whereas the C-terminal mutant was missing amino acids 321-441. Both deletion mutants were expressed under the control of the same promoter and expression system used for the other recombinant tau isoforms. Since the tau mutants were missing portions of the protein that may confer structural stability, a slightly modified purification procedure was developed to minimize the amount of protein loss due to degradation (Figure 38). Fractions from each step of the purification procedure were analyzed by coomassie blue protein stain (Figure 39, panel A) and immunoblot with the N-term antibody (Figure 39, panel B). The induction of the Δ162-209 mutant (arrow) can be clearly seen on both the coomassie stained gel and immunoblot (lane 6), but the induction of the Δ321-441 mutant (arrowhead) was not as defined on the coomassie stained gel (panel A, lane 2). However, the immunoblot indicated that the C-terminal deletion mutant was expressed in the induced cell extract (panel B, lane 2). Comparing the immunoblot of the uninduced samples (lanes 1 and 5) with the induced samples (lanes 2 and 6),
6) revealed some expression of the two mutant proteins in the uninduced fractions. This low level of expression is a common occurrence using this expression system. Subsequent purification steps enriched the mutant proteins as evident by increased staining of the corresponding bands with coomassie blue and the N-term antibody. The deletion mutants were also heat-stable with a majority of the *E. coli* proteins being removed by boiling of the lysed cell extracts (lanes 3 and 7) leaving enriched mutant tau fractions (lanes 4 and 8). Further purification of the tau deletion mutants followed the htau40 purification procedure utilizing ion exchange FPLC. The purified tau mutants were separated on a 10% SDS-PAGE followed by staining with coomassie blue (panel C). The Δ321-441 mutant migrates at a lower molecular weight than the Δ162-209 mutant, which was slightly smaller than the full-length htau40. As seen with all tau isoforms, the apparent molecular weights of the deletion mutants, around 52 and 60 kDa for Δ321-441 and Δ162-209 respectively, were larger than their predicted size based on amino acid sequence.

The two deletion mutants were subjected to *in vitro* phosphorylation by the kinases present in the bovine brain extract and assayed for reactivity with a series of antibodies (Figure 40). Htau40 (lanes 5 and 6) was used as a positive control and only reacted with MPM2 (Panel B), AT8 (Panel C), and pSer396 (Panel D) after phosphorylation (even lanes) as observed previously. Furthermore, both the dephosphorylated (odd lanes) and phosphorylated htau40 were recognized by the BT2 (Panel F) and C-term (Panel E) antibodies. Interestingly, the Δ162-209 mutant
did not shift in molecular weight after phosphorylation (Panel A, lanes 1 and 2), which is normally observed with phosphorylated forms of htau40. On the other hand, the phosphorylated Δ321-441 mutant shifted in molecular weight with the phosphorylated protein migrating as smear (Panel A, lanes 3 and 4). This smearing of the phosphorylated Δ321-441 mutant was most likely due to the loss of the C-terminal region of the protein that also contains additional phosphorylation sites. Furthermore, this shift in molecular size of tau after phosphorylation appears to be primarily due to the phosphorylation of residues located within the N-terminal targeting domain, since the phosphorylation of the Δ162-209 mutant did not produce a similar change in mobility.

To confirm that amino acids deleted from htau40 were indeed removed in each tau mutant, the ability of these mutants to be recognized by antibodies specific for epitopes contained within the missing sequences was examined. The Δ162-209 mutant was assessed for reactivity with the BT2 (Panel F) and AT8 (Panel C) antibodies, which recognize tau epitopes within the corresponding deleted sequence (for epitope locations see Figure 10). Neither the dephosphorylated or phosphorylated Δ162-209 mutant (lanes 1 and 2, respectively) was recognized by either the BT2 or AT8 antibody. On the other hand, the Δ162-209 mutant was reactive with the C-term antibody in both the dephosphorylated and phosphorylated states (Panel E); however, only the phosphorylated form was recognized by pSer396 (Panel D). The Δ321-441 tau mutant, which was missing the entire C-
terminus and approximately two MT binding repeats, was also analyzed for reactivity with the C-term and pSer396 antibodies. Regardless of phosphorylation state, the Δ321-441 mutant was not recognized by either antibody. However, the antibodies to epitopes within the N-terminal flanking region, AT8 and BT2, reacted with this mutant protein in both the phosphorylated and dephosphorylated states, respectively. These results confirmed that the Δ162-209 and Δ321-441 mutants were missing the designated amino acid sequences and indicated that the Δ162-209 mutant was indeed phosphorylated on sites located within the C-terminal flanking region, specifically Ser 396.

The analysis of the phosphorylated forms of both deletion mutants was further examined using the MPM2 antibody. Surprisingly, both deletion mutants reacted with the MPM2 antibody (Panel B) after phosphorylation. The strongest staining was observed with the Δ162-209 mutant (lane 2), but the Δ321-441 mutant was weakly MPM2-reactive (lane 4). The MPM2 reactive Δ321-441 band appeared as a faint smear just above a highly MPM2-reactive band determined to be β-tubulin that was present in the crude kinase extract (Lee and Vandré, unpublished observations; see Figure 22). These results indicated that the MPM2 epitope was not present between amino acids 162-209 due to the intense MPM2 immunoreactivity of the deletion mutant. However, the MPM2 site did not appear to be located between amino acids 321-441 either, since this deletion mutant was weakly MPM2 reactive. Thus, we conclude that there are two possibilities for the
location of the MPM2 epitope on tau: 1) the epitope is located between amino acid 210-237, which contains six additional phosphorylation sites and was present in both deletion mutants, or 2) that there are two MPM2 epitopes on tau varying in degree of reactivity, and the major MPM2 site was eliminated in the Δ321-441 deletion mutant leaving a secondary site that showed weak staining.
DISCUSSION

In the preceding two chapters, PHF-tau and in vitro phosphorylated recombinant full-length human tau (htau40) were shown to be reactive with the MPM2 antibody. The focus of this chapter was to localize the MPM2 epitope on tau by utilizing the in vitro phosphorylation system together with recombinant tau lacking certain regions of the protein. Initially, the htau24 and htau34 isoforms were analyzed for their ability to be phosphorylated on the MPM2 site. Our previous results showed that normal fetal and adult rat tau were not phosphorylated on the MPM2 epitope (Chapter 1, Figure 12); however, kinases normally present in both fetal and adult rat brain could phosphorylate the MPM2 epitope on htau40 (Chapter 2, Figure 29). Because htau40 contains a second N-terminal insert not found in shorter tau isoforms (see Figure 6), it was possible that this second N-terminal insert contained the MPM2 phosphorylation site. Alternatively, the MPM2 epitope could be located in another region of the tau protein and not phosphorylated in the isoforms lacking the N-terminal inserts. This possibility could occur if the N-terminal inserts altered the conformation of the protein such that when it was present tau became a better substrate for the MPM2 epitope kinase. It has been reported that the tau isoforms S3 or S4, which lack the N-terminal inserts were not as readily phosphorylated as those isoforms containing either one or two inserts (Singh et al., 1996). Moreover, the phosphorylation of specific epitopes, such as Ser 396, Ser 404, and the SMI34 site, were phosphorylated at a faster rate, and to a greater
extent, on the tau isoforms containing the N-terminal inserts in comparison to the S3 isoform. These results suggest that the N-terminal inserts acted as modulators regulating the specificity of kinases by altering the conformation of the tau protein or by directly associating with the tau kinases. In the current study, phosphorylated htau24 and htau34 isoforms, containing either zero or one N-terminal insert respectively, were shown to react with the MPM2 antibody to the same extent as the phosphorylated htau40, which contains both N-terminal inserts. This finding indicated that the MPM2 epitope was not located within the second N-terminal insert, nor did the inserts have an affect on MPM2 kinase specificity. Therefore, it appeared that all isoforms of tau were capable of being phosphorylated on the MPM2 site, and the reason that fetal tau is not MPM2 reactive is not simply related to structural differences between the isoforms. The MPM2 phosphorylation site seems to occur in vivo only in the disease state, despite the presence of kinases in normal brain that can phosphorylate this site in vitro.

Since the MPM2 epitope was not located within the N-terminal inserts, another approach was utilized to narrow down the region on tau that contained this site. Two polyclonal antibodies, PTE1 and PMB1, generated against potential MPM2 sequences from topoisomerase II and MAP1B, respectively, were examined for reactivity with PHF-tau and phosphorylated htau40. The recognition of
phosphorylated tau by each of these antibodies further suggested that the MPM2 epitope sequence on tau should have characteristics similar to that predicted by the MPM2 epitope model.

Two potential sites on tau that fit the criteria proposed in the MPM2 epitope model (Ding et al., 1997) were identified as being Ser 198/Ser 199 and Ser 396 (Figure 36). Deletion mutants missing regions spanning both sites were analyzed for MPM2 reactivity after phosphorylation. Unexpectedly, both deletion mutants were capable of being phosphorylated on the MPM2 epitope. The deletion mutant lacking the Ser 198/Ser 199 sites was strongly MPM2 reactive after phosphorylation. In contrast, the mutant missing the region containing the Ser 396 site became only weakly MPM2-reactive.

From these results two conclusions were drawn regarding the location of the MPM2 epitope on tau. First, it is possible that the MPM2 epitope on tau was neither of the predicted sites, but instead was located within another region of tau that was common to both deletion mutants. Even though the reaction with one deletion mutant was weak, the observation that both deletion mutants reacted with the MPM2 antibody after phosphorylation supports this idea. The variation in immunoreactivity of the two mutants can be explained by the fact that the C-terminal deletion mutant migrated as a smear as apposed to a tight band obtained with the
Δ162-209 deletion mutant. Alternatively, more than one MPM2 reactive site may be present on tau, and the weak staining observed with the Δ321-441 deletion mutant reflected reaction to a weaker secondary site.

If the MPM2 site is not present in either deletion mutant than the site must be in a region common to both mutants. The amino acids 210-237 were still present in both the Δ162-209 and Δ321-441 mutants, and this region contains six additional known phosphorylation sites (see Figure 36). All six of these sites, Ser 210, Thr 212, Ser 214, Thr 217, Thr 231, and Ser 235, are phosphorylated in PHF-tau. However, none of these sites fits the proposed MPM2 epitope model (Ding et al., 1997) or a recent refinement of this model (Yaffe et al., 1997). Using a degenerative peptide library an MPM2 consensus sequence was reported that was also consistent with the Ding et al. model (Yaffe et al., 1997). This new model did not take into account any non-proline directed sites such as the epitope define on ERK2. Furthermore, some of the sites present in mitotic proteins, such as wee1, that Yaffe et al. (1997) suggest are reactive with the MPM2 antibody do not fit either model. Thus, both MPM2 epitope models appear to predict only a subset of MPM2-reactive sequences, making it likely that the MPM2 site on tau is different from the consensus sequence predicted in either model. In an attempt to determine if the MPM2 epitope was located between amino acids 210-237, we tried expressing another deletion mutant missing amino acids 162-244, which eliminates both the
Ser 198/Ser 199 and the additional six phosphorylation sites. Unfortunately, this deletion mutant did not express upon induction with IPTG although the plasmid was transformed into the bacteria cells.

If, on the other hand, the weak reaction of MPM2 with the Δ321-441 mutant is due to recognition of a secondary epitope, then the results indicate that the primary MPM2 site is located within the C-terminal region of tau (see Figure 8). Since, the pSer396 and pSer199 antibodies do not have the same phosphorylation profile as the MPM2 antibody (Chapter 2, Figure 26 and 27), it is unlikely that either the major or minor sites are Ser 396 or Ser 199 alone. It still remains to be determined if phosphorylation of residues adjacent to these sites could contribute to antibody recognition. The presence of two MPM2 epitopes on tau is not unrealistic, since other MPM2-reactive proteins such as the MPPs contain more than one potential MPM2 epitope sequence (Westendorf et al., 1994; Matsumoto-Taniura et al., 1996).

To define the MPM2 epitope on tau, a series of site-directed mutants for the residues suggested from the deletion mutant analysis will need to be constructed and examined for their reactivity with the MPM2 antibody after phosphorylation. Furthermore, site-directed mutants eliminating multiple sites would allow determination of the ability of adjacent phosphorylated residues to influence antibody recognition. Comparison of this site on tau with other sites on MPM2-
reactive phosphoproteins would provide a better understanding of the sequence determinants responsible for antibody recognition. Once the MPM2 site on tau is determined, additional questions can be addressed such as: 1) its importance in AD and PHF formation and 2) the kinase(s) and phosphatase(s) that regulate its phosphorylation state and their involvement in AD.
FIGURE 30
Recombinant human tau isoforms
Figure 31. Purification of recombinant human tau isoforms

Both htau24 and htau34 single colonies were grown-up and induced for protein expression with IPTG (see experimental procedures). Samples from several steps of the purification procedure were analyzed for presence of the recombinant tau isoforms by 10% SDS-PAGE stained with coomassie blue (Panel A). Approximately 20 μg of each fraction, determined by BCA assay, was loaded per lane. The steps in the purification scheme analyzed were the uninduced cell extract (lanes 1 and 4), induced cell extract (lanes 2 and 5), and heated supematant (lanes 3 and 6) with the htau24 samples in lanes 4-6 and the htau34 samples in lanes 1-3. The arrowhead indicates the position of htau34 and the arrow indicates the position of htau24.

Panel B is a 10% SDS-PAGE stained with coomassie blue containing approximately 9 μg of purified htau24 (lane 1), htau34 (lane 2), and htau40 (lane 3). The molecular weight markers in both panels are 98, 64, 50, 36, 30, and 16 kDa.
FIGURE 31
Figure 32. Immunoblot analysis of phosphorylated htau24 and htau34.

The ability of htau24 (lanes 1 and 2) and htau34 (lanes 3 and 4) to be phosphorylated on the MPM2 epitope was evaluated and compared to htau40 (lanes 5 and 6). In addition to analysis with the MPM2 antibody (Panel C), the different tau isoforms were also tested for reactivity with the AT8 antibody (Panel B) and the BT2 antibody (Panel D). A coomassie blue protein stain (Panel A) was used as a reference for comparison of the migration patterns of both dephosphorylated (odd lanes) and phosphorylated (even lanes) tau isoforms. Note that all three tau isoforms are phosphorylated by the bovine brain extract and shift in apparent molecular size.
<table>
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<tr>
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<td>QETpPLQPRRR</td>
</tr>
<tr>
<td>3</td>
<td>ALTPPLKGRR</td>
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<td>4</td>
<td>APEALTpPLMKQKGPS</td>
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<td>5</td>
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Potential MPM2 Sequences

topo II peptide    RKEWLTpNFMEDRRRC
MAP1B peptide      LSEFTpEYLSES


FIGURE 33
MPM2 reactive sequences
Figure 34. Immunoreactivity of PHF-tau with polyclonal antibodies PMB1 and PTE1

Approximately 40 µg of PHF-tau was isolated from an AD brain according to the procedure described in Chapter 1. The PHF-tau was separated by 10% SDS-PAGE followed by immunoblot analysis with the MPM2 (lane 1), AT8 (lane 2), PMB1 (lane 3), PTE1 (lane 4), and BT2 (lane 5) antibodies. The concentrations of the antibodies used was 1:500 for MPM2 and AT8, 1:100 for PTE1, 1:50 for PMB1, and 1:250 for BT2. The MW markers are from top to bottom: 98, 64, 50, 36, and 30 kDa.
Figure 35. Immunoreactivity of *in vitro* phosphorylated htau40 with PMB1 and PTE1 polyclonal antibodies.

Htau40 was *in vitro* phosphorylated using a bovine brain extract as in Chapter 2 and analyzed for reactivity with various antibodies after separation by 10% SDS-PAGE. Lane 1 corresponds to dephosphorylated htau40, whereas lane 2 corresponds to phosphorylated htau40. The concentrations of the antibodies used are as follows: 1:1000 for MPM2, 1:500 for AT8, 1:100 for PTE1, 1:50 for PMB1, and 1:250 for BT2. Note that the PMB1 antibody cross-reacts with the dephosphorylated form of htau40 which is due to non-specific reactivity of the crude antiserum (data not presented).
FIGURE 35
MPM2 Epitope Model

\[ \text{NH}_2 \cdots +3 +2 +1 \quad \text{T/S} \quad -1 \quad -2 \quad -3 \cdots \text{COOH} \]

Requirements: phosphorylated Thr/Ser aromatic amino acid at +1, +2, or +3 positions
Enhancement: aromatic or positively charged amino acid at -1, -2, or -3 positions

FIGURE 36
MPM2 epitope model and Potential Sites on Tau
FIGURE 37
Tau deletion mutants
Grow cells to $\text{OD}_{600\text{nm}} \sim 0.6$ $\Rightarrow$ Add IPTG to induce expression of mutant tau proteins

Collect cells by centrifugation and freeze cells with liquid nitrogen

Resuspend cells in 1:1 w/v cold lysis buffer, and lysis cells by 3 cycles of freeze/thaw

Pellet cellular debris by ultra-centrifugation

Boil high-speed supernatant, pellet heat liable proteins, and save tau enriched supernatant $\Rightarrow$ Purify tau proteins via Mono S column chromatography

FIGURE 38
Tau deletion mutant purification scheme
Figure 39. Purification of tau deletion mutants.

Single colonies of Δ162-209 and Δ321-441 tau deletion mutants were grown-up and purified according to the protocol explained in the experimental procedures (see Figure 39 for scheme). Samples from several steps in the purification scheme were analyzed for presence of the deletion mutants by 10% SDS-PAGE followed by either coomassie blue protein stain (Panel A) or an immunoblot with the N-term tau antibody (Panel B) at 1:3000. The N-term antibody recognizes the first 15 amino acids of tau. The samples (20 µg) examined were the uninduced cell extract (lanes 1 and 5), the induced cell extract (lanes 2 and 6), the lysed cells (lanes 3 and 7), and the heated supematant (lanes 4 and 8). Lanes 1-4 correspond to the Δ321-441 deletion mutant (arrowhead, Panel A) whereas lanes 5-8 correspond to the Δ162-209 deletion mutant (arrow, Panel B). The molecular weight markers are 98, 64, 50, 36, and 30 kDa.

Panel B is also a 10% SDS-PAGE stained with coomassie blue containing purified Δ321-441 deletion mutant (lane 1), Δ162-209 deletion mutant (lane 2), and full-length htau40 (lane 3). The molecular weight markers are 98, 64, 50, 36, 30, and 16 kDa.
Figure 40. Immunoblot analysis of *in vitro* phosphorylated tau deletion mutants.

The two tau deletion mutants Δ162-209 (lanes 1 and 2) and Δ321-441 (lanes 3 and 4) were phosphorylated with the bovine brain extract and examined for MPM2 reactivity (Panel B) in comparison to full-length htau40 (lanes 5 and 6). Immunoblot analysis occurred after the phosphorylated samples were first subjected to separation by 10% SDS-PAGE. Both dephosphorylated (odd lanes) and phosphorylated (even lanes) samples were also examined for reactivity with the AT8 (Panel C), pSer396 (Panel D), C-term (Panel E), and BT2 (Panel F) antibody. A coomassie blue protein stain (Panel A) was used to observe the migration pattern of the two deletion mutants in comparison to htau40. Note that the Δ162-209 mutant does not shift in molecular weight after phosphorylation; however, the Δ321-441 mutant does shift in molecular size after phosphorylation.
FIGURE 40
The focus of this thesis was to address one hypothesis for the etiology of Alzheimer's disease (AD), the tau hypothesis. The hyperphosphorylation of tau, a microtubule-associated protein (MAP), is believed to be responsible in part for the degeneration of the hippocampal pyramidal neurons in AD. When tau becomes hyperphosphorylated, it no longer binds to or stabilizes MTs and is released into the cytosol. The inability of tau to associate with the MT network in the affected neuron is believed to result in destabilization of the axonal MTs and subsequent loss of axonal transport along these structures. Failure to transport essential proteins through the axon is thought to cause degeneration of synaptic connections, and subsequently the neuron. In addition, the hyperphosphorylated tau present in the cytosol self-assembles into an abnormal filamentous structure, the paired helical filament (PHF). PHFs aggregate along with other cellular proteins forming an insoluble intracellular deposit called neurofibrillary tangles (NFT), one of the pathological features characteristic of AD. Since 12 of the 19-21 phosphorylation sites on PHF-tau are also phosphorylated on fetal tau, it was speculated that
reactivation of fetal-like and/or mitotic pathways in pyramidal neurons of the adult hippocampus were in part responsible for the hyperphosphorylation of tau and subsequent NFT formation.

Chapter 1 addressed the possibility that the MPM2 antibody, which recognizes a subset of mitotic phosphoproteins, may react with pathological structures present in AD. Several lines of evidence led to this hypothesis. First, MAP1B, which is present in fetal and adult brain, is an MPM2-reactive phosphoprotein (Vandré et al., 1986). Second, MAP1B is also the major MAP present in PC12 cells, which upon differentiation and neurite outgrowth are recognized by the MPM2 antibody (Feng and Vandré, manuscript in preparation). Lastly, phosphorylated forms of MAP1B were found in pyramidal neurons and associated with NFTs (Fischer and Pomano-Clarke, 1991; Ulloa et al., 1994). Thus, we examined the ability of the MPM2 antibody to recognize NFTs in AD brain sections and determined if the phosphorylated form of MAP1B associated with NFTs was MPM2 reactive. Immunohistochemical analysis of AD brain samples showed that the MPM2 antibody did indeed recognize NFTs as well as senile plaques (SP), SP neurites, and neuropil threads (NT), all characteristic AD lesions. Moreover, the MPM2 antibody did not react with the control brain samples. Both the MPM2 and PHF-specific AT8 antibodies colocalized to the same neuropathological lesions suggesting that MPM2 was recognizing a protein associated with PHFs. However, antibodies to MAP1A and MAP1B did not exhibit a similar staining pattern.
as either MPM2 or AT8. This result indicated that the MPM2-reactive phosphoprotein associated with PHFs may not be phosphorylated MAP1B. Unexpectedly, immunoblot analysis showed that PHF-tau isolated from AD brains was recognized by the MPM2 antibody. The MPM2 immunoreactivity could be eliminated by dephosphorylation of PHF-tau with alkaline phosphatase. These results established that PHF-tau contained a mitosis-associated phosphoepitope, and that this epitope was phosphorylated in AD. Interestingly, neither fetal nor adult rat tau reacted with the MPM2 antibody on immunoblots, which further suggested that the MPM2 epitope on tau may be specific for the disease state.

The experiments presented in chapter 2 were carried out to establish whether recombinant human tau, htau40, could be phosphorylated in vitro at the MPM2 epitope site, and to determine the relationship of the MPM2 epitope with other PHF-tau phosphoepitopes. Kinases present in both adult and fetal brain extracts were capable of phosphorylating the MPM2 site, as well as other phosphoepitopes on htau40. The in vitro phosphorylation results indicated a temporal sequence of tau phosphorylation. The Ser 199, Ser 262 and Ser 396 sites were phosphorylated prior to the MPM2 site, and this was followed by phosphorylation of the AT8 epitope at Ser 202 and Thr 205. Moreover, the data was consistent with previous reports showing that phosphorylated tau is a heterogenous population of molecules that are each capable of being phosphorylated on a variety of sites, but any individual molecule may display a
different extent of phosphorylation at each site. In addition, it was shown in Chapter 2 that soluble kinases but not MT-associated kinases were able to phosphorylate the MPM2 epitope on tau; whereas, both soluble and MT-associated kinases were capable of producing the AT8 epitope. These experiments suggested that the AT8 and MPM2 epitopes on tau were regulated by different kinase activities. Additionally, the phosphatase activities regulating these sites also differed, since endogenous and PP2A present in the brain extract could readily dephosphorylated the AT8 site, but the MPM2 site was more stable to phosphatase activity. Taken together, these results suggested that the MPM2 epitope on tau was distinct from Ser 199, Ser 262, Ser 396, and the AT8 site; however, it did not rule out the possibility that the MPM2 epitope could be a dual phosphorylation site like the AT8 epitope. Thus, it was conceivable that the MPM2 epitope could contain either phosphorylated Ser 199 or Ser 396 in addition to an adjacent phosphorylated residue such as Ser 198 or Ser 400.

In chapter 3, the location of the MPM2 epitope on tau was addressed using the in vitro phosphorylation of recombinant tau isoforms and deletion mutants. The smaller isoforms of tau containing either zero or one N-terminal insert were recognized by the MPM2 antibody after phosphorylation in vitro establishing that the MPM2 epitope site was present on all adult tau isoforms. These results also eliminated the known PHF-tau phosphorylation site located at Ser 46 and any sites located within amino acids 45-102 as the MPM2 epitope, since these residues are
missing in the isoforms lacking the N-terminal inserts. Cross-reactivity of the two MPM2-like antibodies, PTE1 and PMB1, with both PHF-tau and in vitro phosphorylated htau40 are consistent with the MPM2 epitope on tau being similar to that predicted by the MPM2 epitope model put forward by Ding et al. (1997). Based upon this model two potential MPM2-reactive epitopes on tau were identified as Ser 198/Ser 199 and Ser 396. However, analysis of deletion mutants missing the corresponding regions showed that both mutants were still capable of being phosphorylated at an MPM2-reactive sites. The MPM2 staining of the Δ162-209 mutant was much stronger than the reactivity of the Δ321-441 mutant. It was concluded from these results that two possibilities exist for the location of the MPM2 epitope on tau. First, the MPM2 epitope could be located between amino acids 210-237, residues that were not missing in either mutant examined. This region contains six additional known phosphorylation sites that are all also present in PHF-tau. Alternatively, there may be a primary MPM2 epitope on tau that is located within the C-terminal region of the protein and a secondary site that was responsible for the weak reaction of MPM2 with the Δ321-441 mutant. A series of site-directed mutants within these regions will need to be constructed, phosphorylated, and assayed for MPM2 reactivity to differentiate between these two possibilities and clearly define the MPM2 epitope site on tau. Determination of the MPM2 epitope would allow for an examination of both the role played by this site in formation of PHFs and the identification of kinase and phosphatase activities that regulate its phosphorylation.
One question that still remains unanswered is why normal fetal and adult rat tau are not MPM2 reactive. One possibility is that the kinase responsible for phosphorylating tau at the MPM2 epitope site is tightly regulated in vivo under normal physiological conditions. It is possible that tau may need to be abnormally phosphorylated prior to phosphorylation of the MPM2 epitope site. The time course experiments in Chapter 2 support this idea, since in vitro phosphorylated htau40 only becomes MPM2 reactive after 6 hours of incubation. In AD, tau is abnormally phosphorylated and does not interact with the MT network, thus allowing redistribution of the protein. The phosphorylation of select Ser/Thr sites that modulate tau-MT interactions may be required prior to phosphorylation of the MPM2 site. The phosphorylation of tau on Ser 262 and Ser 396 has been shown to reduce the ability of tau to bind MTs (Biernat et al., 1993; Bramblett et al., 1993), thereby enhancing the likelihood that tau is redistributed to the cytoplasm. Phosphorylation of the Ser 262 and Ser 396 sites occurs rapidly, within 2 hours of incubation, which is before the phosphorylation of the MPM2 epitope (Figures 26 and 27). The results presented in Chapter 2 also showed that only the soluble kinases, but not the MT-associated kinases, are capable of phosphorylating the MPM2 epitope on htau40 (Figure 28). Taken together, these results are consistent with the possibility that abnormally phosphorylated tau, which is not bound to the MTs, is a substrate for a deregulated soluble MPM2 kinase. Thus, it seems plausible that both the redistribution of tau and the deregulation of the MPM2 kinase may be responsible for the phosphorylation of this site in the disease state. Identification of the
kinase(s) responsible for phosphorylating the MPM2 epitope on tau could provide insight into the altered molecular pathways involved in the progression of AD.

The functional significance of the MPM2 epitope on numerous phosphoproteins has recently been proposed by Yaffe et al. (1997). This group reported that the MPM2 antibody recognizes an amino acid sequence that is similar to the sequence recognized by the catalytic site of the mitotic peptidyl-prolyl isomerase (PPlase) Pin1. Pin1 was first identified by a yeast two-hybrid screen as a protein that interacted with, and inhibited, NIMA kinase (Lu et al., 1996). NIMA kinase is an essential mitotic protein in *Aspergillus nidulans* (Osmani et al., 1991; Lu and Means, 1994). Subsequent work revealed that Pin1 regulates the entry of cells into mitosis and is required for the normal progression of mammalian cells through mitosis (Lu et al., 1996). Moreover, the substrate recognition of Pin1 was identified to be phosphorylation-dependent, and it was suggested that the interaction of Pin1 with mitotic phosphoproteins may act as a conformational switch for the cyclin-dependent kinases (cdk) target proteins (Ranganathan et al., 1997). Interestingly, preliminary evidence also shows that Pin1 interacts with MPM2 phosphoproteins in mitotic HeLa cells (Yaffe et al., 1997). Thus, it was suggested that the MPM2 epitope on these proteins is a Pin1 mitotic regulatory site. Yaffe et al. (1997) proposed that phosphorylation of Ser/Thr-Pro sites by mitotic kinases creates a MPM2/Pin1 recognition site. Subsequent Pin1 catalyzed proline isomerization would induce a conformational change in the phosphoprotein that
could regulate its function. These conformational changes could 1) modulate interactions with other cellular proteins including phosphatases that dephosphorylate the MPM2/Pin1 site, 2) alter the activity and/or localization of the phosphoprotein, or 3) target the protein for degradation.

Pin1 is only one member of a larger class of PPIases that can be subdivided into three families, the cyclophilins, FK506-binding proteins (FKBP), and the parvulin/Pin1 family. Both the cyclophilins, which bind to cyclosporin A, and the FKBP are targets of immunosuppressive drugs; however, their immunosuppressive activity is not related to the inhibition of their PPIase activity. Rather, their immunosuppressive activity is mediated by their ability to inhibit the protein phosphatase calcineurin by forming a complex with the drug and thereby binding calmodulin, a regulator of calcineurin activity. In addition to the PPIase domain, the parvulin/Pin1 family also contains a WW domain, which interacts with short proline-rich sequences within target proteins (Subol, 1996). PPIases were originally thought to function in catalyzing protein folding, trafficking of newly assembled proteins, and regulating fully folded proteins by accelerating isomerization.

In addition to Pin1, cyclophilin A has been implicated in mediating protein-protein interactions in vivo. Interestingly, cyclophilin A is present in the brain and is located in neurons, primarily the pyramidal neurons of the hippocampus and the granule cells of the cerebellum and hippocampus (Göldner and Patrick, 1996).
Staining of hippocampal cell cultures with a cyclophilin antibody localized the protein to the cytoplasm of the cell body and staining extended into the dendrites. The dendritic staining pattern was punctate and appeared in spine-like structures suggesting that cyclophilin A may be present in the post-synaptic regions (Goldner and Patrick, 1996). Recently, another Pin1 isoform, Pin1L was identified by PCR using cDNA from adult temporal cortex and fetal brain libraries (Campbell et al., 1997). The Pin1L isoform lacks the PPIase domain suggesting that it may function strictly as a protein modulator (Campbell et al., 1997). The localization of the two Pin1 isoforms in brain still remains to be determined.

Since Pin1, Pin1L, and cyclophilin A are expressed in adult brain, it is reasonable to speculate that these proteins may interact with the proline-rich domains on neuronal MAPs, such as tau, and modulate their functions. This speculation is not unrealistic in light of data showing that another PPIase, FKBP59-HBI, is partially associated with MTs in several cell lines including mouse neuronal cells (Perrot-Applanat et al., 1995). In addition, FKBP59-HBI is localized to the mitotic apparatus, cleavage furrow, and midbodies during mitosis. Thus, it is possible that PPIases could bind to the proline-rich region of tau's projection domain while this MAP is still associated with the MTs. Sequestration of some PPIases to the MTs could modulate the interaction of tau with other regulatory proteins, such as kinases or phosphatases. Depending on how the PPIase changes the conformation of tau, phosphorylation of select sites on tau, such as Ser 262 or Ser
396, by MT-associated or soluble kinases could be enhanced and subsequently influence the ability of tau to stay bound to the MTs. Alternatively, the phosphorylation of tau on the MPM2 epitope could create a Pin1 recognition site allowing isomerization of the adjacent proline residue and a change in the conformation of tau. This change in conformation may make tau more susceptible to dimerization and formation of PHFs.

In summary, the work presented in this dissertation has established that at least one of the PHF-tau phosphorylation sites is a common mitosis-associated phosphorylation site. The presence of this phosphorylation site on PHF-tau provides additional support for the hypothesis that mitotic and/or fetal-like kinase activities are being reactivated in AD neurons. The ability of the MPM2 site on tau to be phosphorylated in vitro by kinases present in normal brain tissue suggest that the abnormal phosphorylation of tau associated with AD could be partially due to the deregulation of normal kinase and phosphatase activities. Furthermore, it is suggested that the proline isomerase Pin1 may be involved in the formation of PHFs and progression of AD. Identification of the MPM2 epitope on tau would help in determining the role of this tau phosphorylation site the formation of PHFs. Understanding the contribution of the “mitotic” phosphorylation in will aid in determining the underlying molecular mechanism(s) responsible for the development of AD and other neurodegenerative diseases.
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190


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