TRIGGERS AND ENHANCERS OF TAU AGGREGATION: IMPLICATION FOR AD PATHOGENESIS

DISSERTATION

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ABSTRACT

Alzheimer's disease is characterized in part by the aggregation of tau protein into filamentous inclusions. Tau aggregation is not only a robust marker of disease progression, but also contributes directly to degeneration in affected neurons. In this context, the mechanism of tau filament formation and its modulation by posttranslational modification are of fundamental importance.

To clarify the forces that drive neurofibrillary lesion formation, the mechanism of tau filament formation is investigated in vitro and in cellular models.

First, we clarify that the aggregation reaction is triggered by environmental conditions that stabilize assembly-competent conformations. It also shows that Planar aromatic dyes capable of binding the intermediate state with high affinity are also capable of triggering fibrillization. Dye-mediated tau aggregation is characterized in detail and demonstrated as a novel approach to study tau aggregation mechanism in vitro.

Using one of these small molecule dyes as inducer, role of proteolytic posttranslational modification on tau aggregation is studied. The data show that C-terminal proteolysis can modulate tau filament accumulation through decreasing critical concentration and also through directly augmenting the efficiency of the nucleation reaction. Similarly, Congo Red, another planar aromatic dye identified in above
experiment, is applied to tau stable cell line to establish a new cell culture model of tauopathy. Formation of detergent insoluble aggregates is both time and agonist concentration dependent without relating to tau hyperphosphorylation. Results also suggest that conformational changes associated with aggregation are incompatible with microtubule binding, and that aggregation can be toxic in the presence of cellular stress that compromises proteasome function.

Tau hyperphosphorylation precedes neuritic lesion formation in Alzheimer's disease, suggesting it participates in the tau fibrillization reaction pathway. Candidate tau protein kinases include casein kinase 1 (CK1) family, which highly overexpress in Alzheimer's disease brain and colocalize with neuritic and granulovacuolar lesions. Here we show that Ckiδ phosphorylates tau in vivo. Next, we demonstrate that dysbindin structural homologue CK1BP is an isoform-selective binding partner of human casein kinase-1 and that the acidic domain of dysbindin and its paralogs in humans may function to recruit CK1 isoforms to protein complexes involved in multiple biological functions.
DEDICATION

Dedicated to my parents
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LIST OF ABBREVIATIONS

AA, arachidonic acid; Aβ, amyloid β peptide; AD, Alzheimer's disease; ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; Cdk5, cyclin-dependent kinase 5; CHIP, carboxyl terminus of the Hsc70-interacting protein; CK1, casein kinase-1; CK1BP, casein kinase-1 binding protein; Ckiδ-∆317, C-terminal truncated Ckiδ; CR, congo red; DMSO, dimethylsulfoxide; EM, electron microscopy; GSK3β, glycogen synthase kinase-3β; FTDP-17, frontal-temporal dementia with parkinsonism linked to chromosome 17; HEK, human embryonic kidney; His6, polyhistidine tag; IC261, 3-[(2,3,6-trimethoxyphenyl)methylidenyl]-indolin-2-one; IMAC, immobilized metal affinity chromatography; LLS, laser-light scattering; MTs, microtubules; NFT, neurofibrillary tangles; Ni2+-NTA, nickel-nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PHF, paired helical filaments; PIPES, 1,4-piperazinediethanesulfonic acid; siRNA, small interfering RNA; TEM, transmission electron microscopy; ThS, thioflavin S; TR, thiazin red; UPS, ubiquitin proteasome system
CHAPTER 1

1 INTRODUCTION

1.1 Tau in Alzheimer’s disease.

Alzheimer's disease (AD) is the most common form of dementia. It was first reported by Alois Alzheimer, a German physician in 1906. This neurodegenerative disease progressively destroys a patient’s memory and affects their skills of language, recognition, and judgement. Late stage of AD also involves personality and behavioral changes. Although causes of AD are still not very clear, genetic factors and a variety of environmental factors may all contribute to its pathogenesis. AD is characterized by pathological changes which include neuronal loss, reactive gliosis, and especially, the deposition of amyloid plaques and neurofibrillary tangles which are the two hallmarks of Alzheimer’s disease (Iqbal et al., 1989). Amyloid plaques principally consist of beta amyloid protein, while neurofibrillary lesions contains filamentous aggregates (Lee and Rook, 1992) composed of the microtubule-associated protein (MAP) tau in cross-β sheet conformation.

Tau was first identified by its co-purification with tubulin from porcine brain and its ability to promote in vitro microtubule assembly (Cleveland et al., 1977a; Cleveland et al., 1977b; Weingarten et al., 1975). Recombinant tau protein also induces bundling and
elongation of existing microtubules (Brandt and Lee, 1993). Researchers soon confirmed that tau was a major regulator of microtubule formation \textit{in vivo}. Overexpression of wt tau in cells led to microtubule stabilization and bundling, processes formation and extension, and retardation of microtubule-based transport (Ebneth et al., 1998; Lee and Rook, 1992; Leger et al., 1994). In contrast, in tau knockout mice, axonal development delayed (Harada et al., 1994).

For quite a long time, it was uncertain whether the tau aggregation was a pathological result of AD and could only be considered as a diagnostic marker, or it directly contributed to the onset and progression of AD. Recently, people found that various tau missense mutations could cause frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), a rare autosomal dominant neurodegenerative diseases, in which tau pathology is prominent while amyloid pathology is absent (Hutton et al., 1998). These findings clearly established that dysfunction of tau protein was sufficient to cause neurodegeneration and dementia.

Three general hypotheses have been put forward to rationalize a direct implication of tau protein for neuronal degeneration in Alzheimer’s disease. The Loss of function theory argues that in AD, tau protein loses its normal ability of binding and stabilizing microtubules, which results in cell skeleton disruption and neuron degeneration. Several studies support this loss of function hypothesis. For example, tau protein purified from AD brain cannot perform its normal functions such as stabilizing MTs (Bramblett et al., 1993). Moreover, FTDP-17 R406W mutation expressed in cortical cells was highly phosphorylated, resulting in weaker microtubule binding and more cell death compared
with wild type tau (Krishnamurthy and Johnson, 2004). The second hypothesis argues that tau aggregates are directly toxic to cells that harbor them. For example, tau aggregates are capable of inhibiting the ubiquitin-proteasome system (Keck et al., 2003), which may break up cell homeostasis and lead to cellular stress. The third hypothesis is that tau modified post-translationally (such as hyperphosphorylation or truncation) may be directly toxic to cells, which was independent of aggregation of tau. This theory emphasis that aggregated tau is not essential for initial tau toxicity. In stead, under these conditions, aggregation could be protective for cells by sequestering toxic species.

1.2 Tau fibrillization pathway

1.2.1 Fibrillization in vivo.

Although it is quite clear that AD contains tau neurofibrillary lesions, how does tau form filament in the brain is not fully understood. Immunohistochemical and electron microscopic approaches have been used to describe the neurofibrillary lesion formation. Although pathology studies can’t give direct explanation on tau fibrillization mechanism, they provide important in situ information for understanding the biochemical events of AD and also help interpreting experimentally data resulting from cellular or transgenic animal models.

The very beginning of the tau fibrillization is that tau dissociates from microtubules and forms granular nonfilamentous “pretangle” in the cytoplasm. Phosphorylation is thought to effect in this stage not only because it decreases tau’s binding affinity for microtubules, but also because pretangle exhibits epitopes for phospho-tau specific
antibodies, such as AT8 and TG3 (Augustinack et al., 2002; Uchihara et al., 2001). Except for phosphorylation, other factors or certain intracellular environment such as high cholesterol (Ghribi et al., 2006) or oxidation stress (Ko et al., 1997) may also contribute to this stage. Pretangles are Gallyas silver-positive (Sato et al., 2002). The mechanisms of Gallyas silver staining is not clear. What is known is that β-sheet structures are not required for its staining (Gallyas et al., 1980). In contrast, pretangles can not be stained by amyloid-selective fluorescent dyes such as thioflavin-S and Congo red (Galvan et al., 2001), which react specifically to anti-parallel β-sheet fibrils. Since it was hypothesized that a minimum of five adjacent β-sheet strands was necessary for Congo red (Klunk et al., 1989a), the pretangle may be smaller than pentamer. Pretangles also exhibit Alz-50 epitope. This conformation is characterized by the binding between N-termini with the microtubule binding repeat (Carmel et al., 1996).

In the second stage, tau forms mature neurofibrillary tangles inside of neurons. These tangles are principally composed by paired helical filaments (PHF), as well as a small portion of straight filaments (SF) bundles (Kidd, 1963). X-ray diffraction (Wisniewski et al., 1976), electron microscopy (Wischik et al., 1985), Atomic force microscopy (AFM) (Binnig et al., 1986), and fourier transform infrared spectroscopy (Barghorn et al., 2004; Berriman et al., 2003) have been carried out to analysis the structure of PHF and SF. Resulting data suggested that the width of PHF tau filaments ranged from 8 to 20 nm with a regular periodicity of 80 nm (Crowther and Wischik, 1985). Straight filaments has no periodicity and its width is about 10–15 nm (Crowther, 1991). It has been proposed that the formation of NFT within neuron was an ordered
event in which specific secondary and tertiary structures appeared sequentially. During the NFT step, except for heavily phosphorylation, both carboxy and amino truncation events begin to emerge, and can be recognized by another conformation-dependent antibody Tau-66. Thus, it was proposed that during filament formation procedure, tau molecules at least adopted two different conformations including Alz-50 and Tau-66 conformation respectively (Garcia-Sierra et al., 2003). Different from pretangle stage, NFT is Thioflavin-S-positive staining, which indicated that a large amount of anti-parallel beta sheet conformation formed. In addition, ubiquitin begin to integrate in late NFTs (Pei et al., 1998).

The last is the ghost tangle stage, where NFTs survive destruction of the cell and mark its previous location. Ghost tangles still prominently react with Thioflavin-S and Gallyas silver, while losing the AT8 epitope. Ubiquitin is more pronounced in extracellular ghost tangles than in NFTs, and persists even after disappearance of AT8 epitope.

1.2.2 Fibrillization in vitro.

Tau fibrillation in vitro has been widely studied for clarifying tau assembly mechanisms. Generally, purified full length recombinant tau protein, fragments of tau protein essential for aggregation, or soluble PHF-tau purified from AD brain were used in such researches. Since tau protein is highly soluble and can’t efficiently form filament spontaneously even in high concentration, inducers for tau fibrillation are added in the in vitro assembly assay. Heparin, polyglutamate, nucleic acids, fatty acids, alkyl sulfate
detergents, anionic microspheres have been used to induce tau fibrillization in vitro. Their abilities to induce tau aggregation depend on the negative charges. Comparing with heparin and polyglutamate, fatty acids and alkyl sulfate detergents act in micellar form and provide an anionic surfaces (Chirita and Kuret, 2004; Necula et al., 2003) for tau fibrillization. They promote tau fibrillization more efficiently under near physiological conditions using physical level of tau protein concentration (Wilson and Binder, 1997). Moreover, unlike heparin or RNA, which is sequestered from tau protein in vivo and couldn’t act as an in vivo inducer, anionic micelles mimic cellular membranes which could provide intracellular anionic surfaces (Chirita et al., 2003). Thus they have been used widely for studying tau assembly pathway in vitro. Tau fibrillization have been analyzed using quantitative electron microscopy (Necula and Kuret, 2004a), fluorescence spectroscopy (Friedhoff et al., 1998a; King et al., 1999), static light scattering (Abrahah et al., 2000), sedimentation (Alonso et al., 2001; Chirita et al., 2003; Li et al., 2002; Perez et al., 2002), and intrinsic tau fluorescence assays (Li et al., 2002; Luo et al., 2000). These methods complement to each other and were used for different purposes.

Whatever inducer was used, tau in vitro aggregation pathway was quite similar and its progression followed a sigmoidal curve with a clear lag time, an exponential growth, and an equilibrium phase. It consists of two basic reactions: a nucleation reaction followed by an elongation reaction. In nucleation reaction, tau monomer associates with each other and forms “nucleus” (Necula and Kuret, 2004c). This reaction is energetically unfavorable, and is the rate-limiting step for the whole procedure, which is reflected by the lag time in the sigmoidal curve. A minimum (critical) concentration of
tau is required for nucleation reaction (Necula and Kuret, 2004c). Critical concentrations vary for each inducer due to their different potency. Once nucleus is formed, elongation reaction will proceed spontaneously due to the energetically favorable property of this reaction. Some factors are capable to accelerate this reaction such as phosphorylation and glycation (Necula and Kuret, 2004b).

Previously, recombinant tau monomer was considered as the substrate of the nucleation reaction. Later, people noticed that tau monomer by itself is highly soluble, it can not spontaneously form nucleus in its physiological concentration. Nucleation reaction can proceed only after adding inducers. Researches demonstrated that inducers such as anionic microspheres transformed tau molecule into assembly compatible intermediates which constituted the real substrates of the nucleation reaction (Chirita and Kuret, 2004).

Although there has been progress in the understanding of tau aggregation mechanism, many questions left unanswered. It is not clear about the actual size of the tau nucleus, although it has been estimated as 8–14 protomers (Barghorn and Mandelkow, 2002). It is also not clear about the conformational changes in each step. Whether dimer or oligomer is the unit for elongation reaction is not known. Further studies on tau in vitro fibrillization are needed to answer these questions.

1.3 Post-translational modification.

The tau that accumulates in disease lesions differs from recombinant protein in its state of posttranslational modification, including levels of phosphorylation (Buee et al.,
2000), glycation (Ledesma et al., 1995), proteolytic truncation (Gamblin et al., 2003; Mena et al., 1996; Rissman et al., 2004), racemization (Watanabe et al., 1999), and nitration (Horiguchi et al., 2003). Here, I will focus on two of them: phosphorylation and c-terminal truncation.

1.3.1 Role of tau phosphorylation.

There are 45 serine, 35 threonine, and 5 tyrosine residues in the longest isoform of human tau protein which contains 441 amino acids. Therefore tau is a good substrate for phosphorylation. In 1977, Cleveland and others found that tau was phosphorylated by a protein kinase which was co-purified with microtubules (Cleveland et al., 1977a). Later, it was found that one mol normal tau protein carried 1.9 mol phosphates. In contrast, PHF-tau has a significantly higher stoichiometry of phosphorylation which is 6-8 mol phosphate/mol protein (Ksiezak-Reding et al., 1992). Over 30 phosphorylation sites have been identified in PHF-tau, while much fewer found in normal tau and fetal tau. The hyperphosphorylation of tau in AD is either due to the overexpression of protein kinases such as GSK3β, Cdk5, PKA, or due to the decrease of protein phosphatases such as PP1, PP2A and PP2B. Moreover, tau hyperphosphorylation can also be regulated indirectly by other factors such as Pin1 (Hamdane et al., 2006), Aβ (Wang et al., 2006), and oxidation (Ko et al., 1997).

Although most researchers agree that tau is a phospho-protein in vivo, the roles of tau phosphorylation in the pathogenesis of AD are not fully understood. To answer this
question, several aspects including when tau hyperphosphorylation occurs during the progression of AD, what its pathological consequences are and how it contributes to tau aggregation should be considered.

1.3.1.1 Tau hyperphosphorylation is the early event in the pathogenesis of AD.

More and more data indicated that tau hyperphosphorylation was the early event in the pathogenesis of AD, preceding the filament formation and neurodegeneration. Most of these data came from immunocytochemistry studies of tau phosphorylation in different stages of neurofibrillary degeneration. Phospho-tau was found not only existing in tangles but also in the neurons lacking neurofibrillary tangles (Bancher et al., 1989). The cyclic AMP-dependent protein kinase phosphorylated htau40 on Ser214 and Ser409 (Jicha et al., 1999a; Zheng-Fischhofer et al., 1998). Positive immunostaining probed by antibodies recognizing phosphorylations on these two sites accumulated in the early PHF aggregates. Thus tau phosphorylation may be the early step for normal tau converting to aggregates (Jicha et al., 1999b). Similarly, AT8 antibody, which recognizes tau phosphorylated at Ser202 and Thr205, immunoreacted with neurons exhibiting initial cytoskeleton changes but lacking of aggregations. Thus, phosphorylated on these sites may promote an early cytoskeleton change (Braak et al., 1994). Moreover, the active forms of both MEK1/2 and ERK1/2 co-localized with pretangles. With neurofibrillary tangles developing, they begun to emerge in those large aggregates (Pei et al., 2002). Taken together, all of these in situ data provided evidences that tau hyperphosphorylation was an early event of AD.
It is interesting to note that tau phosphorylation pattern varies with the severity of AD. It has been reported that the pretangle was phosphorylated at serines 199, 202 and 409; NFT was additionally phosphorylated at serine 396, 422 and threonine 231; and “ghost tangles” was phosphorylated mainly at serine 396 (Kimura et al., 1996). In a parallel work, reactivities of 11 phospho-specific tau antibodies were examined in several AD cases. They distributed differently in terms of stages of neurofibrillary tangle development. Different from above data, antibodies TG3 (pT231) reacted with pretangle instead of NFT (Augustinack et al., 2002). This disagreement may result from different standard for staging AD samples. These data demonstrated that tau phosphorylation at different sites may have different implications for altering tau conformation and tau toxicity. Moreover, protein kinases usually contact and regulate each other, and sometimes work in a cascade to regulate tau phosphorylation. Drosophila PAR-1 kinase directly phosphorylated tau at S262 and S356, which was a prerequisite for tau phosphorylation by GSK-3 and Cdk5 on several other sites (Nishimura et al., 2004). These findings suggest that tau phosphorylation in AD is very complex.

1.3.1.2 Pathological consequences of tau hyperphosphorylation.

Comparing with staging hyperphosphorylation in the progression in AD, more controversies exist about their pathological functions. Although tau phosphorylation is generally believed able to cause microtubule disruption and neurodegeneration, some researchers consider it as a protective mechanism maintaining cell’s stability. Both of these opinions are discussed below.
1.3.1.2.1 Opinion 1: tau hyperphosphorylation is toxic to neurons.

In 1984, it was demonstrated that after treating with alkaline phosphatase, tau promoted polymerization of microtubules more rapidly (Lindwall and Cole, 1984). After that, more and more researchers believe that hyperphosphorylation on specific sites promotes dissociation of tau from microtubules, which leads to microtubule destabilization. This may be the most well-defined role of tau phosphorylation up to date.

Although a large number of protein kinases are capable to phosphorylate tau in vitro, far less contribute to tau hyperphosphorylation in vivo. PKA, CK1, GSK3β, and Cdk5 were found associating with microtubules. Thus they are in the close vicinity to phosphorylate tau in vivo (Flaherty et al., 2000). Tau was hyperphosphorylated and reduced its affinity for microtubules in 3T3 and CHO cells expressing tau and GSK3β. Microtubule bundling also reduced in these cells (Wagner et al., 1996). There was evidence that phosphorylation on a specific primed site, Thr231, was responsible for these GSK3β-mediated effects (Cho and Johnson, 2004). Cdk5 is another major kinase that phosphorylates tau in vivo. It was reported that Cdk5/p39 phosphorylated tau at Ser202 and Thr205, which led to a reduction of its affinity for microtubules (Takahashi et al., 2003). Similarly, in transgenic mice overexpressing human p25, another activator of Cdk5, tau was hyperphosphorylated and the cytoskeleton disrupted. The p25 transgenic mice also displayed anxiety-like behavior (Ahlijanian et al., 2000). Except for serine and threonine, tyrosine residues are also phosphorylated in AD. Mass spectrometry revealed the phosphorylation at Tyr-394 in PHF-tau, which was demonstrated as the main residue for Abl (Derkinderen et al., 2005). Also, it was reported that src family tyrosine kinase
fyn phosphorylated tau at Tyr18 (Lee et al., 2004). Co-transfection of tau and fyn in NIH3T3 cells reorganized the microtubule array (Lee et al., 1998). Abnormal phosphorylation of the cytoskeleton could result in neurodegeneration. P25/Cdk5 hyperphosphorylated tau in vivo, decreased tau's binding to microtubules, and induced morphological changes and apoptosis (Patrick et al., 1999). Phosphorylated tau may also act as the toxic moiety independent of tau aggregation. Tet/GSK3β mice exhibited most pathological features of AD including tau hyperphosphorylation, neuron death, reactive gliosis, and deficits in spatial memory without formation of tau aggregates (Hernandez et al., 2002). Pseudohyperphosphorylated tau expressed in PC12 cells and in neurons reduced tau-microtubule association and destabilized the microtubule network. Moreover, apoptosis was induced in the absence of tau aggregates. This data supported the hypothesis of "toxic gain of function" of soluble tau (Fath et al., 2002).

1.3.1.2.2 Opinion 2: tau hyperphosphorylation is protective to neurons.

Traditional conception about tau phosphorylation in the AD pathology dominated for many years. However contradictory opinions emerged recently. It was reported that MARK and PKA phosphorylated several sites both within and flanking the repeats domain of tau protein and led to reduced affinity for microtubules. Surprisingly, the phosphorylation at Ser214 inhibited tau assembly (Schneider et al., 1999). Moreover, tau phosphorylation may be one of the protective mechanisms that neurons eliminate the toxic species. Phosphorylation of tau by GSK3β was necessary for its recognition by the E3 Ub ligase CHIP and the E2 conjugating enzyme. Thus tau phosphorylation is
important for its degradation through ubiquitination (Shimura et al., 2004). A protective role of tau phosphorylation was further supported by the fact that pseudophosphorylated at S422 (S422E) was more resistant to proteolysis by caspase 3, whose activity was essential for the formation of tau c-terminal truncations which were more prone to assembly (Guillozet-Bongaarts et al., 2006). Although more data are needed to prove the protective role of tau phosphorylation, this theory provides an alternate view for pathology of AD.

1.3.1.3 **Tau phosphorylation alone does not sufficiently trigger tau aggregation.**

Tau hyperphosphorylation and tau aggregation are the two major features of AD, FTDP-17 and all other tauopathies. Therefore an obvious question is: what’s the relation between them? Since tau hyperphosphorylation is considered preceding tau aggregation (which is reviewed in above chapters), there is a possibility that tau phosphorylation triggers tau aggregation. However, most recent data antagonized this assumption. First, although tau phosphorylation lowers its isoelectric point, which may lower its solubility, phosphorylated tau can’t form filaments spontaneously \textit{in vitro}. Second, tau phosphorylated at Ser262 and Ser214 by PKA \textit{in vitro} was reported to inhibit tau assembly (Schneider et al., 1999). Third, pseudophosphorylation study clarified that phosphorylation modification was not capable to trigger tau fibrillization, but rather enhanced the reaction through lowering its critical concentration. Thus the reaction equilibrium was shifted toward the aggregation state (Necula and Kuret, 2004b). Further study showed that tau phosphorylation could also stabilize the filaments once they were formed (Necula and Kuret, 2005). Last, several transgenic mice and cell culture models
overexpressing protein kinase only led to tau hyperphosphorylation without resulting in tau aggregation. Taken together, these data demonstrate that tau phosphorylation alone can’t sufficiently trigger tau aggregation. In stead, it may enhance tau fibrillization either by lower its critical concentrations for assembly, or by translocating tau from microtubules to cytosol, which leads to increased intracellular substrate for fibrillization.

1.3.2 Role of c-terminus proteolysis.

A Monoclonal antibody (mAb) 423 was the first antibody generated against the pronase resistant core of the Alzheimer PHF, which made it possible to distinguish between normal soluble tau and PHF-associated tau protein in the brain tissues. Mapping of the mAb 423 epitope indicated that minimal protease resistant unit of the core PHF was 93-95 residues long, and c-terminal truncation at Glu391 was critical for mAb 423’s immunoreactivity. Since mAb 423 labels intracellular neurofibrillary tangles, dystrophic neurites and granulovacuolar degeneration in Alzheimer's disease (Bondareff et al., 1990, 1991; Mena et al., 1991), it has been proposed that the C-terminal truncation of tau detected by mAb 423 reflects an endogenous event in PHF assembly, although the endogenous protease which cleave tau at this site is still not been identified yet.

Tau truncated at D^{421} has also been reported. Monoclonal antibody, Tau-C3, was generated to specifically recognize this c-terminal truncation form of tau. This antibody stains NFTs, neuropil threads and dystrophic neurites in AD brain, the three most common pathological exhibitions of in situ tau aggregation. Thus at least some of pathological aggregates contain truncated tau. Caspase and calpain have been identified
as the endogenous proteases which cleave tau at D^{421} \textit{in vivo}. Phosphorylation at S^{422} inhibited the proteolysis at D^{421} by caspase 3. In contrast, the cleavage was facilitated in neurons treated with Abeta peptide.

The truncation state of tau influences many of its pathologic characteristics. Both type of c-terminal truncations have been shown to assembly more easily than the full-length tau \textit{in vitro}. In contrast, a synthetic peptide containing the c-terminal fragment (tau 422-441) inhibited tau assembly. The author demonstrated that peptide adopted an \( \alpha \)-helical conformation and interacted with residues 321-375 in the microtubule binding repeat region, which led to the inhibition of tau polymerization.

NMR spectroscopy study of the hydrophobic C-terminal segment of tau showed that it adopted alpha-helix conformation with a stabilizing C-capping motif. Cleavage of tau at D^{421} by caspase-3 will remove the C-capping motif, which will expose the C-terminal \( \alpha \)-helix to cellular carboxypeptidases, leading to progressive unwinding of the helix. Thus, the protein is more prone to further proteolysis, generating more fragments during apoptosis.

Tau c-terminal truncations are not only produced during apoptosis, but also considered as inducers of apoptosis in human neuronal cell lines, such as SH-SY5Y and NT-2 cells. Further mapping of apoptotic properties of tau fragments in COS cells indicated that the longest fragment with full apoptotic capacity was tau l-422. These results showed that the absence of the 19 C-terminal residues of tau caused a dramatic change in the properties of the protein, turning it into an apoptotic effector.
Cleavage also has been used for staging the evolution of AD progression. However, there are still disagreements on whether truncation of tau proteins emerges before or after it assembles into filament. Raúl Mena et al found that formation of mAB423 epitope occurred in the early pathological stage in which only amorphous non-fibrillar structure demonstrated. In contrast, most recent studies showed that Tau-C3 and mAb 423 recognized mature tangles, while pre-tangle neurons, in which fibrillar structures were not evident in light or fluorescent microscopy, were preferentially labeled by Alz50 alone.

1.4 Cellular model of tau aggregation.

Non-neuronal cells, neuronal cells and primary neuron culture have been used to model tauopathy. The advantages of non-neuronal cell lines are that they are easy to be transfected and that there are no disturbances of endogenous tau, which simplify data analysis. Thus non-neuronal cells are ideal for examining the biochemical properties of human tau protein, such as the implications of its phosphorylation, truncation and mutation for aggregation and microtubule dynamics. Neuronal cell lines are similar with non-neuronal cells in the sense of easy handling and maintenance. However, since they have the potential to differentiate into post-mitotic neurons under certain conditions, they are more appropriated to study how tau involved in the physiological processes, the pathological events, as well as morphological changes of AD. Another advantage is that exogenous fibrillization inducers can be tested in neuronal cells with a physiological concentration of tau. Although neurons are the host cells of tauopathy, few AD cellular
models have been made in primary neuron cultures because they are difficult to be transfected. In addition, obtaining and maintaining of consistently pure and healthy neuron cultures are both time-consuming and expensive.

In AD, tau protein is hyperphosphorylated and aggregates into filaments, microtubules disrupt and neurons degenerate. An ideal cellular tauopathy model would include all of these features. In reality, most existing cellular models emphasize on one of these pathological changes. Since tau aggregations is the hallmark of AD and has been assumed as the key of the AD progress, only those models bearing tau aggregation are discussed in this chapter. Basically, 4 approaches have been tried to induce tau aggregation in cells.

1.4.1 Approach 1: overexpressing susceptibility genes for AD.

Molecular genetics studies have shown that polymorphic variation in the APOE gene is a genetic risk factor for late onset AD (Saunders et al., 1993). ApoE is proteolytically cleaved in AD brains and the resulting c-terminal-truncated fragments of apoE accumulate and are toxic to neurons. Expression of c-terminal truncated forms of apoE4 (Δ272-299) induced NFT-like inclusions in cultured mouse primary cortical neuron (Huang et al., 2001). Tau protein found in these intracellular inclusions was phosphorylated. Although these findings were also true in human neuronal NT2-N cells and mouse neuroblastoma (Neuro-2a) cells, neurofibrillary tangles were not induced in tau-expressed non-neuronal cells such as CHO, COS-7, C-6, HepG2, and McA-RH7777 (Ljungberg et al., 2002).
Gene encoded amyloid precursor protein (APP) was also found linked to AD (Tanzi et al., 1987). In AD, APP is proteolytically processed into beta-amyloid peptides. These peptides include Aβ40 and -42, the major components of amyloid plaques in AD. SH-SY5Y cells stably expressing wild-type and mutant forms of human tau were exposed to aggregated synthetic Aβ42. This caused the formation of PHF-like filaments in these cells. However, tau was not present in the RIPA-insoluble pellets when Ser-422 was mutated to alanine or glutamic acid, suggesting phosphorylation on this site may be essential for the filament formation (Ferrari et al., 2003). Similarly, cultured hippocampal neurons obtained from wild-type, tau knockout, and human tau transgenic mice were treated with pre-aggregated Aβ (1-40) peptide. Morphological changes including neurite degeneration and neuron death presented in neurons expressing either mouse or human tau proteins, but not in tau-depleted neurons (Rapoport et al., 2002). Although this research explored the potential role of tau in the Abeta-induced neurodegeneration in the central nervous system, it didn’t address the issue that whether Aβ peptide treatment induced tau aggregations in these neurons.

1.4.2 Approach 2: Overexpressing kinases or inhibiting phosphatases to hyperphosphorylate tau.

Although hyperphosphorylation of tau protein have been widely studied in many cell lines, only few of these researches resulted in tau aggregates in cells. Similarly, hyperphosphorylated tau cannot spontaneously form filaments in vitro. Thus, although
tau hyperphosphorylation may facilitate aggregate formation, it is not an efficient approach to make cellular model of tau aggregation without cooperating with other approaches.

Oligodendroglia cell line overexpressing Htau40 was treated with okadaic acid (OA), an inhibitor of protein phosphatase 2A. After the treatment, tau was hyperphosphorylated and lost its affinity to microtubules. Simultaneously, tau aggregates formed which could be stained by thioflavin-S. However, these aggregates were not stable and were degraded within 24 hours. Proteasome inhibition stabilized these aggregates and led to fibrillary deposits (Goldbaum et al., 2003).

A similarly research has been done using SH-SY5Y human neuroblastoma cells. In addition to treating the cell by OA alone, cells also were treated by OA together with 4-hydroxynonenal (HNE), a product of oxidative stress, which has ability to induce fibrillization of phosphorylated tau but not unmodified tau in vitro. Okadaic acid alone only led to tau hyperphosphorylation, while tau aggregates were found in cells treated with both okadaic acid and HNE (Perez et al., 2002). These findings suggest that phosphorylation alone is not sufficient to induce tau filament formation in cells.

In contrast, another cellular model demonstrated that the aberrant phosphorylation of tau led to the formation of short fibrils which were RIPA-insoluble. Instead of just co-expressing one specific kinase together with tau as many similar researches did, an adenovirus-mediated gene expression system was used to synergistically express tau, ∆MEKK, JNK3, and GSK-3β in COS-7 cells. Tau was phosphorylated at multiple sites,
and formed aggregates that were detergent-insoluble and thioflavin-S positive and displayed relatively short fibrils about 10-nm diameters which were believed as tau oligomers (Sato et al., 2002).

1.4.3 Approach 3: overexpressing FTDP-17 mutations.

FTDP-17 is a presenile dementia affecting the frontal and temporal cortex and some subcortical nuclei. Its symptoms include behavioral and personality changes and Parkinsonism (Poorkaj et al., 1998). This autosomal dominant hereditary neurodegenerative disorder was caused by various mutations occurred on in exons and introns of the tau gene. The findings that FTDP-17 mutations altered the levels of specific tau isoforms and promoted tau aggregation in the brain (D'Souza et al., 1999; Hong et al., 1998) led to the notion of modeling tauopathy in cells by overexpressing these FTDP-17 mutations. And this approach seems to be the most efficient way to induce tau aggregation in cells till now.

Overexpression of wild type tau in Sf9 cells led to the formation of long processes in cytoplasm which resembled the axons. EM revealed that microtubule bundles existed in these processes and with the same polarity orientation (Knops et al., 1991). In contrast, overexpression of V337M mutation disrupted microtubules and reduced the number of microtubules per process. This result implicated that normal function of tau was essential for microtubule stabilization and bundling (Frappier et al., 1999). In addition, insect Sf9 cells were also infected with the longest isoform of tau protein simultaneously carrying
three FTDP-17 mutations (G272V, P301L and R406W). Tau filaments resembling PHFs were found in the sarkosyl-insoluble extract of the Sf9 cells (Gomez-Ramos et al., 2004).

Tetracycline-off inducible expressions of 4-repeat wild-type tau and the corresponding mutants V337M and R406W were performed in human neuroglioma H4. The formation of “particulate tau” was found after ultra-centrifugation. Overexpression of R406W gave the highest amount of sarkosyl-insoluble tau and it was the only one whose insoluble fraction was thioflavin S-positive and contained 15- to 5-nm-wide filaments (DeTure et al., 2002). This inducible system was also used to express simultaneously three isoforms of tau (4R0N, 3R1N, and 4R1N) encoded by a tau minigene construct in human neuroblastoma BE(2)-M17D cells. Filamentous tau aggregates formed in cells were sarkosyl-insoluble, and were confirmed by immunoelectron microscopic examination and thioflavin S binding assay (Ko et al., 2004).

FTDP-17 mutations also were stably expressed in CHO cell lines individually. Delta K is the only one which resulted in fibrillar aggregates when these mutations were expressed individually. Similar results were found when a triple tau mutant containing V337M, P301L, and R406W was stably transfected in CHO cell line. The formation of aggregated also correlated with reduced affinity to bind microtubules (Vogelsberg-Ragaglia et al., 2000).

1.4.4 Approach 4: overexpressing wild type tau protein.

It is very hard to induce tau aggregation by overexpressing wild type tau in cells since tau protein is highly soluble and can’t form filament spontaneously in vitro. Years
of application of this approach didn’t lead to many positive results. One established model based on this strategy took the advantage of the lamprey reticulospinal neurons which could be easily injected and maintained \textit{in situ}. Microinjection of plasmids encoding human tau protein into these neurons \textit{in situ} induced tau 'straight filaments' (Hall et al., 1997). This model has been used to study the phosphorylation, microtubule and synapse loss, and \textit{in vivo} assembly stages of tau aggregation, and also to validate the inhibitory function of a compound which was identified through \textit{in vitro} study (Hall, 1999; Hall et al., 2000; Hall et al., 2002; Hall et al., 2001).

1.5 Summary.

The kinetic pathways through which natively unfolded tau protein adopts higher order structure and fibrillizes have important implications for clarifying the pathogenesis of tauopathies such as AD. Experiments performed \textit{in situ} and \textit{in vitro} suggest key roles for posttranslational modification such as phosphorylation and c-terminal truncation in tau aggregation pathway. A challenge for the future is to rationalize the many observations regarding stages of lesion formation and tau posttranslational modification \textit{in vivo} in the context of aggregation kinetics derived for experimentation \textit{in vitro}. In addition to clarifying mechanism of tau pathogenesis, such information will lead to improved cellular models of disease and to the development of inhibitory ligands with potential therapeutic utility.
CHAPTER 2

2 TRIGGERS OF FULL-LENGTH TAU AGGREGATION: A ROLE FOR PARTIALLY-FOLDED INTERMEDIATES

2.1 Introduction.

Tau is a microtubule-associated protein implicated in the progression of AD\(^1\) and other tauopathic neurodegenerative diseases (Buee et al., 2000). It purifies from normal brain tissue as an ensemble of alternative splice products with monomeric quaternary structure (Cleveland et al., 1977a). In disease, however, tau protein accumulates in lesions composed of fibrillar aggregates displaying the cross-beta sheet diffraction pattern of “amyloid” (Berriman et al., 2003). In vitro, tau can be induced to assemble with sigmoidal kinetics consistent with a nucleation-dependent process (Chirita et al., 2004; Necula and Kuret, 2004c). This behavior is typically ascribed to the requirement for a necessary but scarce species in the reaction pathway termed the “nucleus” (Ferrone, 1999). Once formed, however, subsequent lengthening of the nascent filament by stepwise addition of protomeric species is energetically favorable and follows in a reaction termed elongation. The favorable elongation reaction relative to the less favorable nucleation reaction results in the characteristic features of this mechanism, including a pre-equilibrium phase characterized by a time lag during which nucleation proceeds, the existence of a critical protomer concentration, below which filament
formation is not supported, and seeding behavior, whereby the addition of preformed nuclei or filaments surmounts the energy barrier of nucleation. Experience with recombinant tau constructs suggests, however, that classic nucleation-elongation theory as elaborated for spontaneous self-association (i.e., homogeneous nucleation) is insufficient to completely account for the aggregation behavior of tau. For example, recombinant full-length tau produced \textit{in vitro} or through expression \textit{in situ} does not spontaneously assemble as predicted by theory, even at high levels of supersaturation (Crowther et al., 1994; Friedhoff et al., 1998a; King et al., 1999). Moreover, the predicted seeding behavior is inefficient in the absence of exogenous fibrillization promoters (Friedhoff et al., 1998b; King et al., 1999).

These data suggest that molecular events in addition to those underlying classic nucleation-elongation behavior are responsible for triggering the fibrillization of full-length tau isoforms. Because tau is posttranslationally modified in disease, abnormal phosphorylation, glycation, and/or oxidation reactions have all been suggested to play triggering roles. Experience with phosphorylation mimicry mutants in the context of purified recombinant tau preparations indicates, however, that although modifications can promote fibrillization, the mechanism is limited to enhancement of the elongation reaction through lowering of the critical concentration for assembly (Necula and Kuret, 2004b). Similar results were found for glycated recombinant tau preparations (Necula and Kuret, 2004b). Moreover, oxidation of tau into disulfide-bonded dimers can accelerate fibrillization without triggering it (Barghorn and Mandelkow, 2002), and both N- and C-terminal proteolytic truncations are later-stage events in disease temporally
unrelated to the triggering reaction (Horowitz et al., 2004). In fact, the ultrastructure of tau filaments in AD tissue suggests that the trigger may not involve spontaneous self association at all. In end stage disease, individual PHFs appear in endwise association with membranes, consistent with surface-mediated nucleation and unidirectional extension from stable tau-membrane complexes (Gray et al., 1987). These data are consistent with a heterogeneous nucleation mechanism, where association of tau with other intracellular components triggers aggregation. This reaction can be mimicked in vitro by contacting purified preparations of full-length recombinant tau protein with polyanions or anionic micelles, vesicles, or microspheres, with fibrillation proceeding over a period of hours (Chirita and Kuret, 2004; Chirita et al., 2003; Perez et al., 2002). Immediately after contact, and well before filament formation, species of tau form that can be detected by ThS, a non-covalent probe of β-sheet structure (Chirita and Kuret, 2004). We have termed these species “intermediates” because of the timing of their appearance during the reaction time course, and have proposed that they are the true substrates for the nucleation reaction. This interpretation predicts that tau fibrillization is triggered by conditions that support the adoption of assembly-competent conformations.

Here we test this hypothesis using full-length recombinant tau protein and anionic inducers of assembly. The data indicate that the stabilization of an intermediate folding state characterized by increased β-sheet structure is sufficient to trigger the fibrillization of full-length tau protein.
2.2 Material and methods.

Materials. Recombinant His-tagged htau40 was prepared as described previously (Carmel et al., 1996; Necula et al., 2003). AA (Cayman Chemicals, Ann Arbor, MI) was dissolved in ethanol (333 mM) and stored at -80°C until used. Glutaraldehyde, uranyl acetate, and 300 mesh carbon-coated copper grids were from Electron Microscopy Sciences (Ft. Washington, PA). Stock solutions of thiazin red (TCI America, Portland, OR) and ThS (Sigma, St. Louis, MO) were prepared in water, whereas Congo Red (City Chemical, West Haven, CT) and ANS (Sigma, St. Louis, MO) were prepared in DMSO and ethanol, respectively. Carboxylate-conjugated polystyrene microspheres (90 nm diameter, molecular area = 12 Å²/eq) were from Bangs Laboratories, Inc (Fishers, IN).

Fibrillization assays. Under standard conditions, htau40 was incubated without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) at 37°C for up to 24 h in the presence or absence of fibrillation inducers (AA, carboxylate modified microspheres, ThS, Congo Red, thiazin red, or ANS). For analysis by EM, aliquots were removed, treated with 2% glutaraldehyde (final concentration), mounted on formvar/carbon-coated 300 mesh grids, and negatively stained with 2% uranyl acetate as described previously (Chirita et al., 2003; King et al., 1999). Random images were viewed in a Phillips CM 12 transmission electron microscope operated at 65 kV, captured on film at 8,000 - 60,000-fold magnification, digitized, and imported into Optimas 6.5.1 for quantification of filament lengths and numbers (King et al., 1999). Individual filaments ≥ 50 nm in length were counted manually.
**ThS fluorescence measurements.** Tau was aggregated at 37°C as described above except that the reactions contained 10 µM ThS. Resultant changes in fluorescence were monitored at λ_{ex} = 440 nm and λ_{em} = 495 nm in a FlexStation plate reader (Molecular Devices, Sunnyvale, CA) operated at sensitivity 10, high PMT using black-matrix, clear-bottom 96-well isoplates (Wallac, Turku, Finland) sealed with transparent foil (NUNC; Denmark).

**Circular dichroism.** Samples were prepared for CD analysis by desalting (Bio-Rad Econo-Pac 10DG column) into 100 mM NaClO₄, 10 mM H₃BO₃, pH 7.4. These buffer components were employed because of their transparency at far UV wavelengths (Schmid, 1997). Spectra were collected (186 – 260 nm) at 25 °C with an AVIV model 202 CD spectrometer and a quartz cuvette with 1 mm path length. Four repetitive scans (4 s integration time, 1 nm step size, and 1 nm bandwidth) were recorded, averaged, and corrected for buffer-only blank without additional filtering or smoothing. Raw CD signals (in millidegrees) were converted to mean residue molar ellipticity [θ]_M (with units of degree·cm²/dmol) using the formula [θ]_M = [θ]_{obs}/(10·l·c·n), where [θ]_{obs} is the observed ellipticity in millidegrees, l is the pathlength in centimeters, c is the molar concentration of protein, and n is the number of residues in the protein (Woody, 1996). Secondary structure composition of α helix (normal and distorted), β-strand (normal and distorted), turns, and random coil were estimated from net spectra using CONTIN/LL (Provencher and Glockner, 1981), CDSSTR (Krishnamurthy and Johnson, 2004), and SELCON3 (Sreerama et al., 1999) algorithms. Resultant estimates were then averaged and presented ± S.D. (Sreerama and Woody, 2000).
Hydrodynamic Analysis. Analytical size-exclusion chromatography was performed (4°C) on a 24-mL (10 x 300 mm) Superose 6 10/300GL column equilibrated with buffer 10 mM HEPES, pH 7.0, 100 mM NaCl and operated at 0.5 mL/min. Standards (Potschka, 1987) included thyroglobulin (8.0 nm), equine apoferritin (6.2 nm), yeast alcohol dehydrogenase (4.6 nm), and bovine serum albumin (3.6 nm). Hydrodynamic radii were estimated from partition coefficients as described previously (Vancura et al., 1993).

Velocity sedimentation measurements were performed in linear 4 - 20% sucrose gradients containing 10 mM HEPES, pH 7.4, 150 mM NaCl. Standards included bovine serum albumin (4.3 s), bovine erythrocyte carbonic anhydrase (3.2 s), myoglobin (1.9 s), and cytochrome C (1.6 s). After centrifugation (200,000 g for 20 h at 4°C), gradients were fractionated and sedimentation coefficients were estimated as described previously (Vancura et al., 1993). Native molecular weight was calculated from hydrodynamic parameters as described previously (Siegel and Monty, 1966) using a partial specific volume calculated from tau amino acid sequence (Perkins, 1986). Theoretical hydrodynamic radii for tau in native, molten globule, pre-molten globule, random coil, and denatured states were calculated from molecular weight using the empirical equations deduced by Tcherkasskaya et al. (Tcherkasskaya et al., 2003). Observed (i.e., apparent) frictional ratios \((f/f_0)_{obs}\) were calculated from hydrodynamic radii \(r_{hyd}\) by the equation (Siegel and Monty, 1966):
\[(f/f_0)_{\text{obs}} = r_{\text{hyd}} \left[ (3\nu M/4\pi N)^{1/3} \right] \]  

(1)

where \( \nu \) is the partial specific volume calculated as described above, \( M \) is the molecular weight, and \( N \) is Avogadro’s number.

**Analytical Methods.** Sigmoidal reaction progress curves were fit to a 3-parameter Gompertz function as described previously (Necula and Kuret, 2004c). Lag times, defined as the time when the tangent to the point of maximum polymerization rate intersects the abscissa of the sigmoidal curve (Evans et al., 1995), were calculated from the resultant Gompertz parameters (Necula and Kuret, 2004c).

Hyperbolic reaction progress curves were fit to the polynomial series (Philo and Selwyn, 1973):

\[ y = \alpha + \beta t + \gamma t^2 + \ldots + \zeta t^6 \]  

(2)

where \( y \) is fluorescence intensity at time \( t \), and the coefficient \( \beta \) approximates the initial velocity.

Concentration-response data were fit to a three-parameter log normal function:

\[
y = ae^{-0.5 \left( \ln \left( \frac{x}{x_0} \right) \right)^2} \left( \frac{\ln \left( \frac{x}{x_0} \right)}{b} \right)^2 \]

(3)

where \( y \) is the amount of product at ligand concentration \( x \), \( a \) is the maximum amount of product appearing at optimal concentration \( x_0 \), and \( b \) is a constant.
Nucleation statistics were fit to a Poisson distribution:

\[ P(n) = \frac{e^{-\lambda} \lambda^n}{n!} \]  

(4)

where \( P(n) \) is the probability of a microsphere randomly nucleating \( n \) filaments when the average number of filaments per microsphere is \( \lambda \).

2.3 Results.

2.3.1 Recombinant tau protein is an extended monomer in solution.

Authentic tissue-derived tau proteins are asymmetric monomers when free in solution (Cleveland et al., 1977a). Conversely, it has been claimed that recombinant tau forms dimers at high concentration (Barghorn and Mandelkow, 2002) in a reaction that is further favored by the presence of a poly-His tag (Yao et al., 2003). Dimerization has been postulated as a key pre-nucleation step in tau aggregation (Barghorn and Mandelkow, 2002). In view of these conflicting claims, recombinant htau40 was subjected to hydrodynamic analysis using a combination of gel filtration chromatography and velocity sedimentation. Results show that under the near-physiological buffer conditions used for fibrillization experiments, recombinant htau40 is an asymmetric monomer with hydrodynamic properties similar to authentic tau (compare Table 2.1 with Ref. 2).

Empirical formulae relating hydrodynamic radius to chain length have been derived for different folding states (Tcherkasskaya et al., 2003). The utility of these equations for comparing proteins of differing chain lengths can be increased by
converting hydrodynamic radii into frictional ratios \((f/f_0)_{\text{obs}}\) as described in Experimental Procedures. Comparison of the experimentally determined \((f/f_0)_{\text{obs}}\) value for recombinant poly-His-tagged human htau40 with these theoretical predictions indicated that the preparation consists of a monomer in random coil conformational state at the start of the aggregation pathway (Figure 2.1), consistent with the behavior of tissue-derived porcine tau (Cleveland et al., 1977a) and recombinant non-tagged human htau40 (Paudel, 1997).

2.3.2 **Surface-mediated intermediate formation is first order with respect to tau concentration.**

Monomer progresses to polymer through an intermediate stage characterized by an ability to bind ThS, a fluorescent probe of \(\beta\)-sheet structure (Chirita and Kuret, 2004). Because ThS fluorescence appeared immediately during fibrillization lag time and also below the critical concentration of filament formation as assessed by electron microscopy and laser light scattering measurements, it was assumed to directly reflect the concentration of non-filamentous species. To further characterize these species, the time course of ThS fluorescence resulting from incubation of monomeric htau40 in the presence of carboxylated microspheres was followed in real time. ThS fluorescence appeared immediately upon contacting the microspheres and then increased hyperbolically as a function of time (Figure 2.2A). In contrast, preincubation of tau with microspheres prior to addition of probe resulted in immediate formation of ThS fluorescence, whereas incubation of tau with ThS alone (at low micromolar
concentration) yielded only modest fluorescence (data not shown). These data confirmed that intermediates formed after contacting the microsphere surface and were not induced by the presence of low micromolar concentrations of probe.

Previously, intermediate formation was shown to be first order with respect to microsphere concentration (Chirita and Kuret, 2004). To determine reaction order with respect to tau protein, the initial velocity of ThS fluorescence production was measured as a function of tau concentration. Because initial velocities were estimated during fibrillization lag time, the rate of appearance of ThS fluorescence was assumed to reflect the formation of prefibrillar species. Replots of this data in double log format revealed a slope of 0.90 ± 0.02, indicating that the appearance of ThS fluorescence is first order with respect to tau protein concentration (Figure 2.2B). These data suggest that the intermediate corresponds to a conformation of monomer. It further suggests that aggregation of tau into supramolecular complexes occurs after intermediate formation.

Nucleation-elongation reactions are characterized by a critical concentration, above which all additional protomers incorporate into filaments (Zhao and Moore, 2003). It is the highest protein monomer concentration that does not support fibrillization, and therefore corresponds to the abscissa intercept of the tau concentration dependence curve (Timasheff, 1981). To determine the relationship between intermediate formation and filament critical concentration, the monomer concentration dependence of htau40 fibrillization and intermediate formation was determined in the presence of carboxylated microspheres using EM and ThS fluorescence assays, respectively. Fibrillization was directly proportional to tau concentration with an abscissa intercept of 2.06 ± 0.10 μM
(Figure 2.3). This value is consistent with estimates made by laser light scattering methods in the presence of anionic surfactants (Necula and Kuret, 2004b; Necula and Kuret, 2004c) and indicates that the final equilibrium between filament ends and unincorporated tau is similar in the presence of diverse anionic inducers. The relationship between final ThS fluorescence intensity at equilibrium and tau concentration below the critical concentration for fibrillization also was linear under these conditions (Figure 2.3). In contrast to filament formation, however, the curve intersected the abscissa within one standard error of the estimate of the origin (0.03 ± 0.05 µM) (Figure 2.3) indicating that intermediate formation did not have a critical concentration. These data are consistent with the ThS-reactive intermediate being a monomer that does not require a minimal concentration for formation.

2.3.3 Intermediate secondary structure.

The ability of the tau intermediate to bind ThS probe suggests it contains increased β-sheet structure relative to natively unfolded recombinant starting material. To test this prediction, htau40 was incubated in the presence or absence of arachidonic acid inducer for 20 min and then subjected to circular dichroism spectroscopy. The analysis was conducted at the critical concentration and within fibrillization lag time to ensure that mature filaments did not contribute to circular dichroism signal. In the absence of arachidonic acid, full-length htau40 yielded a CD spectrum typical of unfolded proteins, with a broad minimum of ellipticity centered at 195 nm and a shoulder at 220 nm (Figure 2.4A). Molar ellipticities at 200 and 222 nm were -13538 and -3236 respectively,
consistent with theoretical studies showing that the coiled state can accommodate substantial local secondary structure (Fitzkee and Rose, 2004). The presence of arachidonic acid led to an increase in the \([\theta]_{222}/[\theta]_{200}\) ratio, consistent with intermediate formation being accompanied by an increase in secondary structure and a loss in random coil (Figure 2.4A). The final ellipticity values at 222 and 200 nm were characteristic of the pre-molten globule folding state (Uversky and Fink, 2004). The amounts of secondary structure were estimated by fitting CD spectra to three independent algorithms as described in Experimental Procedures (Figure 2.4B). In the absence of inducer, htau40 adopted 5.5 ± 1.4 % alpha helix, 25.0 ± 3.9 % beta strand, and 69.4 ± 3.6 % random coil/turn conformation. These values were generally similar to those found at room temperature for bovine tau isolated without harsh heat or acid treatments (Ruben et al., 1997). Inducer converted an additional 7.1 ± 1.2 % of residues to beta conformation at the expense of random coil/turn conformation. Because the proportion of the preparation in partially folded conformation is unknown, this increase must be considered a lower limit. Apparent increases in alpha helix content were not significant at \(p < 0.05\). These data suggest that the tau intermediate is a partially folded monomer enriched in \(\beta\)-sheet content relative to the starting conformation.

### 2.3.4 Intermediate tertiary structure.

In addition to its secondary structure signature, the pre-molten globule state is characterized by a partially collapsed structure with a loosely packed hydrophobic core. To confirm that the intermediate had partially folded character, htau40 was incubated in
the presence or absence of arachidonic acid inducer for 24 h and then examined for an ability to bind ANS, a fluorescent probe of surface exposed hydrophobic patches (Chaffotte et al., 1997; Schonbrunn et al., 2000; Semisotnov et al., 1991). The analysis was conducted below the critical concentration to ensure that filaments did not contribute to ANS fluorescence. ANS in buffer alone fluoresced weakly at optimum wavelength of $\lambda_{em} = 500$ nm. In contrast, ANS in the presence of intermediates (prepared by incubation of htau40 with AA inducer) fluoresced brightly with a blue-shifted optimum of $\lambda_{em} = 487$ nm (Figure 2.5). The increase in fluorescence intensity was not fully recapitulated by either tau protein alone or by AA micelles formed from incubation with protamine, indicating that the enhanced fluorescent signal observed in the presence of tau and AA came primarily from binding to altered conformations of tau (Figure 2.5). Taken together, the above data suggest that anionic inducers stabilize monomeric tau in a partially folded, $\beta$-sheet enriched conformation, and that this conformation precedes the polymerization reaction.

### 2.3.5 Intermediate stabilization triggers tau fibrillization.

We hypothesize that formation of the intermediate conformation is necessary and sufficient for triggering fibrillization. If so, then conditions that stabilize the intermediate should induce tau fibrillization. To test this prediction, htau40 was incubated with increasing concentrations of ThS, a dye that binds tightly ($K_D \sim 1 \mu M$) to the intermediate conformation (Chirita and Kuret, 2004; King et al., 1999). Other dyes known to interact with $\beta$-structure were investigated as well, including Congo Red (Klunk et al., 1989b)
and thiazin red (Mena et al., 1995). Linkage theory (Wyman and Gill, 1990) predicted that rising dye concentrations would drive tau monomer equilibrium toward the intermediate conformation by mass action, and then toward the filamentous state through positive polysteric linkage with the self-association reaction (linkage refers to the influence of ligand binding upon other equilibria including aggregation reactions and phase transitions). Results confirmed that Congo Red, thiazin red, and ThS were all capable of inducing tau fibrillization independently of anionic inducers (Figure 2.6). Dye-induced filaments differed from anion-induced filaments in morphology, growing as twisted ribbons with maximum widths of 25 ± 4 nm, minimum widths of 11 ± 2 nm, and half periodicity of 170 ± 39 nm \( (n = 42) \) during time periods \( \leq 24 \) h. In contrast, straight filaments formed over identical time periods with arachidonic acid had a consistent width of 13 ± 2 nm \( (n = 45) \) and no twist (Figure 2.6). Moreover, dye-induced filaments were greater in number and shorter in length than arachidonic acid induced filaments, suggesting that dyes were especially efficacious inducers of nucleation. Although capable of binding the partially folded structure of the intermediate, ANS did not induce fibrillization at concentrations up to 500 \( \mu \)M, indicating that the linkage reaction was selective for anionic planar aromatic dyes known to bind \( \beta \)-sheet structure (Figure 2.6). Congo Red was the most potent and efficacious inducer tested, with concentrations as low as 10 \( \mu \)M yielding large numbers of small filaments (Figure 2.6). ThS treatment also led to large numbers of small filaments, but its potency was at least 10-fold lower than Congo Red. Thiazin red was the least efficacious ligand tested, yielding few filaments of relatively long length at optimal concentrations. Nonetheless, these
filaments were most amenable to experimentation (because they were fewer in number and longer in length) and so thiazin red was used for detailed characterization experiments described below.

To quantify the potency of the reaction, htau40 was incubated with varying concentrations of thiazin red and assayed for fibrillization using quantitative EM. Inducer activity followed a biphasic log-normal concentration response curve with an optimum potency of 114 ± 2 µM and 115 ± 3 µM in the presence of 1 and 2 µM htau40, respectively (Figure 2.7). Congo Red followed a similar profile, although the concentration optimum appeared ~10-fold lower (data not shown). These data indicate that, unlike anionic inducers, dye-mediated fibrillization reactions do not depend on tau/inducer ratios.

2.3.6 Intermediate stabilization promotes filament nucleation.

To determine whether thiazin red influenced filament nucleation rates, the time course of htau40 fibrillization was followed by EM over a period of up to 24 h in the presence of 50 and 100 µM thiazin red at 37°C and no agitation. When incubated at htau40 concentrations ≤1 µM, reaction progress curves were sigmoidal with clear lag, exponential growth, and equilibrium phases (Figure 2.8). As thiazin red concentrations were raised from 50 to 100 µM in the presence of 0.8 µM htau40, lag times decreased from 0.73 ± 0.09 h to 0.30 ± 0.04 h (Figure 2.8). These data suggest that acceleration of tau fibrillization with dye inducers results from increased rates of filament nucleation.
To confirm this observation, tau fibrillization induced by anionic microspheres was examined in the presence and absence of thiazin red. Nucleation events were counted as the number of filaments formed on the surface of each microsphere (Figure 2.9A). In the absence of thiazin red ~80% of beads had no filaments, ~20% had one filament (Figure 2.9A), and a small percentage had 2 filaments (Figure 2.9C). Beads with three or more filaments were never observed ($n = 100$). The data fit a Poisson distribution (averaging 0.19 filaments/microsphere) indicating that nucleation events were random and independent of each other. Addition of thiazin red to 50 µM concentration shifted the Poisson distribution so that the average microsphere contained 1.95 filaments (Figure 2.9B,C). Further increase in thiazin red concentrations to 100 µM raised the average still higher to 2.17 filaments/microsphere. Together these data suggest that intermediate stabilization had a large positive effect on nucleation rate, and that the effect was additive with anionic inducers.

2.3.7 Effects of intermediate stabilization on critical concentration.

In the presence of thiazin red, htau40 fibrillized at submicromolar concentration, far below the critical concentration estimated in the presence of anionic inducers. To clarify this issue, critical concentrations were estimated as a function of thiazin red concentration. Results showed extremely low critical concentrations of 0.20 ± 0.07 and 0.21 ± 0.05 at 50 and 100 µM thiazin red concentrations, respectively (Figure 2.10). These data indicate that thiazin red treatment led to critical concentrations 10-fold lower than did anionic inducers, and that the presence of dyes influenced post-nuclear equilibria in addition to nucleation rate and filament morphology.
2.3.8 **Thiazin red induces fibrillization in monomeric form.**

Although anionic inducers such as arachidonic acid are monomers below 200 µM in aqueous solution, the presence of protein such as tau triggers the rapid formation of micellar aggregates in which fibrillization inducing activity resides (Chirita et al., 2003). Planar aromatic dyes also self-assemble to form supramolecular aggregates at high concentration (Stopa et al., 1998; Stopa et al., 2003), suggesting that formation of anionic micelles may also mediate the dye inducer activity. To test this hypothesis, the absorbance properties of thiazin red were measured in the presence and absence of tau protein. Dye aggregation is accompanied by characteristic shifts in absorbance optima depending on the structure of the aggregate formed (Murakami, 2002). In methanol, where dye aggregation is not supported, thiazin red absorbance was characterized by a major peak centered at 514 nm and a shoulder at 548 nm (**Figure 2.11**). This pattern is characteristic of hydrazone tautomers of 1-naphthol-2-phenylazo derivatives such as thiazin red (Antonov et al., 1999; Antonov and Stoyanov, 1995; Iijima et al., 1998). The pattern did not change significantly in the presence of aqueous buffer with or without tau protein (**Figure 2.11**). These data suggest that thiazin red does not form detectable supramolecular complexes under assay conditions, and that the active form of the dye is a planar monomer.
2.4 Discussion.

2.4.1 Triggers of tau fibrillization.

Isolated tau proteins have the hydrodynamic properties of random coil monomers in solution, consistent with their equilibrium conformation being “natively unfolded”. Despite the lack of organized tertiary structure, tau monomers do not assemble spontaneously under physiological conditions over experimentally tractable time periods. The barrier to aggregation can be overcome *in vitro* through truncation of the tau molecule to short peptides within the microtubule-binding repeat region (the region that mediates fibrillation *in vivo*). Indeed, peptide fragments ranging from as small as two amino acids (Goux et al., 2004) to as large as four full repeats (Arrasate et al., 1999; Perez et al., 2002; Schweers et al., 1995) aggregate spontaneously to form amyloid filaments over a period of days when incubated at high concentration (>150 µM). Spontaneous assembly becomes progressively less efficient as the length of tau increases toward full-length isoforms (Schweers et al., 1995), eventually requiring extremes of ionic strength, pH, temperature, and tau concentrations (Crowther et al., 1994). At near physiological buffer conditions and tau concentrations (1-10 µM), aggregation of full-length isoforms becomes still less favorable and virtually undetectable over a period of weeks (King et al., 1999). These data suggest that the rate limiting barrier for aggregation of full-length tau isoforms is conformational in nature.

Although truncation reduces the conformational barrier to aggregation, the macroscopic pathway observed in authentic tissue indicates that the initial steps of aggregation involve primarily full-length tau isoforms (Horowitz et al., 2004). What
triggers fibrillization of full-length tau under physiological conditions? In the case of anionic inducers it appears to be the stabilization of monomeric intermediates characterized by increased beta structure (as detected by circular dichroism and binding of fluorescent probe ThS), and partially collapsed tertiary structure (as detected by fluorescent probe ANS) resembling a premolten globule state. Similar states mediate the fibrillization of α-synuclein and amylin (Kayed et al., 1999; Uversky et al., 2001a; Uversky et al., 2001b), suggesting commonality in the mechanism of fibrillization of natively unfolded proteins. However, not all intermediates may be thioflavin dye reactive, and not all reactive intermediates are monomers. For example, aggregation of transthyretin monomer initiated by shifting supersaturated solutions to acidic pH yields immediate formation of thioflavin dye reactivity as found here for tau protein (Hurshman et al., 2004). In the case of transthyretin, however, thioflavin dye reactivity appeared in tandem with protein oligomers rather than monomers. Consistent with the reactive species being multimeric, the initial velocity of thioflavin dye fluorescence increased as a power function of protein concentration. These data indicate that the intermediates involved in amyloid formation may be structurally diverse.

In the current work, tau assembly intermediates appeared in the presence of anionic surfactants and microspheres, but it is likely that other tau fibrillization inducers act similarly. For example, treatment of tau with the polyanion inducer heparin modulates its ability to serve as a substrate for protein kinases, indicating a change in conformation upon binding (Paudel and Li, 1999). In addition, solvent additives such as urea that induce fibrillization of full-length tau proteins (Montejo de Garcini and Avila,
1987; Montejo de Garcini et al., 1988) also modulate the formation of assembly-competent conformations of insulin (Ahmad et al., 2004). In both cases, activity resides with urea but not guanidine. Together these data are consistent with the stabilization of assembly-competent intermediate conformations being intimately connected with the triggering of fibrillization.

The conversion from natively unfolded tau to assembly competent intermediate has the characteristics of an allosteric transition. Initially, natively unfolded htau40 is an extended monomer containing ~25% of residues in beta strand conformation. In the presence of anionic surfactant-inducer, htau40 adopts a condensed conformation containing additional beta conformation. In addition to anionic surfactants, tau fibrillization can be induced by planar anionic aromatic ligands complementary to β-sheet structure such as Congo Red, thiazin red, and ThS. In contrast, the nonplanar anionic aromatic dye ANS, which has affinity for condensed folding states including premolten globule but not for β-sheet structure (Kamen and Woody, 2001), did not induce tau fibrillization even at concentrations up to 500 µM. These data suggest that the formation of additional β-sheet structure in the intermediate is a key requirement for triggering the nucleation reaction. It cannot be excluded, however, that certain structural elements interfere with nucleation and are resolved by intermediate formation.

2.4.2 The tau aggregation pathway.

Once β-sheet enriched intermediates are populated, tau aggregates spontaneously in a reaction characterized by a lag time and critical concentration. In terms of aggregation
kinetics, intermediate formation leads to shorter aggregation lag times reflecting increased rates of nucleation. Because intermediate formation is rapid and intramolecular, oligomerization (including dimer formation) appears to be a secondary reaction potentially related to nucleation.

How does intermediate stabilization promote filament nucleation? Hypothetical models of amyloid fibrils constructed from fiber diffraction patterns provide potential clues. Intramolecular-refolding models (Perutz et al., 2002), where amino acid residues of a single protein protomer form parallel and cylindrical $\beta$-sheets, are consistent with monomeric nucleus cluster sizes determined from nucleation rates (Gamblin et al., 2003). In this case, the rate-limiting intramolecular conformational change is necessary and sufficient for the elongation reaction to proceed spontaneously. In other words, intramolecular refolding is coincident with the nucleation reaction. In contrast, the intermolecular-association of $\beta$-sheets predicted by zipper-spine models (Ivanova et al., 2004) suggests that nucleation of this structure involves protein oligomerization to form a stable unit cell before elongation is supported. Because the tau nucleus is oligomeric (Friedhoff et al., 1998b), tau filaments are better modeled by the zipper-spine model than the intramolecular-folding model put forth for proteins containing poly-L-glutamine tracts. We propose that tau nucleation corresponds to an oligomerization reaction involving face-to-face packing of beta sheet segments arranged orthogonal to the fiber axis (Diaz-Avalos et al., 2003; Ivanova et al., 2004). Seen in this way, one major role of intermediate formation is to prearrange $\beta$-sheet structure for incorporation into the oligomeric nucleus. Once nucleated, the nascent filament lengthens by addition of
protomers parallel to the filament axis. Tissue-derived tau filaments achieve a final $\beta$-sheet content of $57 \pm 7\%$ (Berriman et al., 2003). This value, which is an average from mixed isoforms and presumably overestimated owing to limited proteolysis (Horowitz et al., 2004), suggests that $\beta$-structure extends far beyond the ~90 amino-acid residue core of paired helical filaments (Novak et al., 1993). These data are consistent with the progression from natively unfolded full-length monomer to intermediate and finally filament conformation being characterized by continuously increasing $\beta$-sheet content.

In vitro, tau filament morphology varies with isoform (Hasegawa et al., 1997) and primary structure (Perez et al., 2002). The data presented herein indicates that morphology also depends on the nature of inducer. Anionic surfactants and microspheres induce primarily untwisted 13-nm wide filaments from htau40 over early time periods, and these transition to twisted filaments with the mass per unit length of paired helical filaments over a period of days (King et al., 2001). In contrast, thiazin-red induced twisted ribbons with maximal width of 25 nm from the earliest time points. The factors influencing amyloid filament morphology are not fully understood. In the cases of $\beta2$-microglobulin (Perez et al., 2002) and immunoglobulin light chain variable domain (Khurana et al., 2001), filament morphology is linked to the conformational properties of the precursor state. Differences tau filament morphology in the presence of different inducers may reflect similar considerations. In fact, it is conceivable that nucleation rate, filament morphology, and critical concentration all depend on the nature of the fibrillization inducer owing in part to differences in the fine structures of the intermediate species they stabilize. Alternatively, dyes may incorporate into filaments and thereby
influence morphology at other stages of assembly. Because tau filaments develop over a period of decades (Morsch et al., 1999), the final conformation of filaments *in vivo* (i.e., paired helical filaments) may reflect free energy considerations (Turner et al., 2003) rather than just the nature of the inducer or structure of the assembly intermediate.

### 2.4.3 Triggers and enhancers.

What triggers fibrillization of full-length tau isoforms in sporadic disease? The endwise association of tau filaments with intracellular membranes in biopsy specimens of AD tissue (Gray et al., 1987) suggest that anionic surfaces may present pathophysiologically relevant sources of triggering activity. But as shown here, planar aromatic dyes also can trigger the fibrillization reaction, perhaps mimicking the activity of naturally occurring small molecules (Santa-Maria et al., 2004). Indeed, small-molecule metabolites can induce the fibrillization of proteins other than tau (Goldbaum et al., 2003), suggesting additional sources of exogenous triggering activity. Moreover, tissue-derived tau contains multiple posttranslational modifications, and these too may trigger the aggregation reaction. For example, phospho-tau isolated from AD brain aggregates spontaneously *in vitro* in a reaction that is antagonized by phosphatase treatment (Alonso et al., 2001). However, these experiments require pretreatment of tau with urea, a known modulator of intermediate structure (Ahmad et al., 2004). In fact, both phosphorylation mimicry and glycation enhance tau fibrillization at the step of elongation (Necula and Kuret, 2004b). Acting in this way, enhancers stabilize filaments and increase the driving force for nucleation without necessarily triggering the reaction.
In terms of the zipper spine model, triggers and enhancers are predicted to differentially affect the equilibria orthogonal and parallel to the filament axis, respectively. It is likely, therefore, that alternative splicing, mutation, and posttranslational modifications will have differential effects on the nucleation and extension phases of the tau aggregation reaction. It will be useful to characterize these modulators at the level of intermediate formation.

2.4.4 Pharmacological considerations.

Tau filament formation is a robust marker of degeneration, and so is an attractive target for premortem diagnostic development. Small-molecule ligands capable of binding amyloid conformation with high affinity may be useful for this purpose (Zhuang et al., 2003). The data presented here, however, suggest that reagents such as these must be used with care owing to their ability to drive the fibrillization reaction. The high concentrations (>25 μM) required for dyes such as ThS to drive fibrillization suggest the existence of a preequilibrium, where binding to natively unfolded tau is slow because few binding sites are prearranged. It will be important to determine whether binding affinity for filaments can be separated from affinity for intermediate states, or whether linkage with the nucleation reaction can be severed.
2.4.5 Conclusions.

Protein aggregation is controlled by both conformational and colloidal stability in solution, either of which can be rate limiting for amyloid formation depending on conditions (Ferrari et al., 2003). Despite being natively unfolded, similar considerations apply to tau protein. Under physiological conditions, the equilibrium conformation of full-length htau40 is not assembly competent, and so the fibrillization reaction is triggered by those agents or mutations that stabilize assembly competent conformations. Triggering activity is characterized by greatly accelerated nucleation rates. In contrast, posttranslational modifications that stabilize the filamentous state by promoting the elongation reaction act as enhancers. These are characterized by decreases in critical concentration without acceleration of the nucleation reaction. Evolving models of amyloid structure suggest a physical basis to these observations.

2.5 Summary.

Alzheimer’s disease is characterized in part by the accumulation of full-length tau proteins into intracellular filamentous inclusions. To clarify the events that trigger lesion formation, the aggregation of recombinant full-length four repeat tau (htau40) was examined in vitro under near-physiological conditions using transmission electron microscopy and spectroscopy methods. In the absence of exogenous inducers, tau protein behaved as an assembly incompetent monomer with little tertiary structure. The addition of anionic inducers led to fibrillization with nucleation-dependent kinetics. On the basis of circular dichroism spectroscopy and reactivity with thioflavin S and 8-anilo-1-
naphthalenesulphonic acid fluorescent probes, inducer stabilized a monomeric species with the folding characteristics of a pre-molten globule state. Planar aromatic dyes capable of binding the intermediate state with high affinity were also capable of triggering fibrillization in the absence of other inducers. Dye-mediated aggregation was characterized by concentration dependent decreases in lag time, indicating increased nucleation rates, and submicromolar critical concentrations, indicating a final equilibrium that favored the filamentous state. The data suggest that the rate limiting barrier for filament formation from full-length tau is conformational, and that the aggregation reaction is triggered by environmental conditions that stabilize assembly competent conformations.
### Table 2.1. Recombinant htau40 Physical Properties

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic Radius (nm)</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>Sedimentation coefficient ($s_{20, w}$)</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Native molecular mass (Da)$^a$</td>
<td>45,000 ± 8,000</td>
</tr>
<tr>
<td>Native molecular mass (Da)$^b$</td>
<td>47,881</td>
</tr>
<tr>
<td>Native structure</td>
<td>Monomer</td>
</tr>
<tr>
<td>($f/f_0$)$_{obs}$</td>
<td>2.32 ± 0.39</td>
</tr>
<tr>
<td>($f/f_0$)$_{shape}$</td>
<td>1.95 ± 0.33$^c$</td>
</tr>
<tr>
<td>Axial ratio (prolate ellipsoid)</td>
<td>~19:1</td>
</tr>
</tbody>
</table>

$^a$Estimated from hydrodynamic parameters

$^b$Estimated by mass spectroscopy

$^c$Corrected for hydration assuming 0.5 g water/g protein
2.7 Figures.

Figure 2.1. Tau protein is a monomer with random coil character.

The \((f/f_0)_{\text{obs}}\) (observed frictional ratio) estimated for recombinant poly-His tagged htau40 by hydrodynamic methods (○; Table 2.1) was compared to theoretical values (■) for the protein in six folding states (1, native; 2, molten globule; 3, pre-molten globule; 4, random coil; 5, denatured in 8 M urea; and 6, denatured in 6 M guanidine HCl) calculated (by equation 1) on the basis of empirical correlations between hydrodynamic radius and chain length (Tcherkasskaya et al., 2003). Each folding state is depicted as a category arbitrarily placed at equal intervals on the abscissa, whereas the solid line is drawn solely to aid visualization. The relative errors for calculated \((f/f_0)_{\text{obs}}\) values are reportedly ≤10% (Tcherkasskaya et al., 2003). The hydrodynamic behavior of the tau preparation used throughout this work was characteristic of a monomer in random coil state.

50
Figure 2.2. Intermediate formation is first-order with respect to tau concentration.

(A) The time course of ThS fluorescence formation resulting from incubation (37°C) of htau40 at 0.5 (□), 1 (■), 2 (○), and 4 µM (●) in the presence of carboxylate-substituted polystyrene microspheres (90 nm diameter, 11.6 Å²/eq molecular area; 124 pM) was followed by fluorescence spectroscopy. Each point represents a fluorescence measurement at time $t$, whereas solid lines represent best fit of the data points to a sixth power polynomial series. The rate and extent of ThS fluorescence increased with increasing tau concentrations.

(B) Replot of the initial velocities ± SE estimated from the progress curves shown in Panel A versus tau concentration in double logarithmic format, where the line represents best fit of data points to a linear regression. The slope of the replot (0.90 ± 0.02) was consistent with the initial velocity of ThS signal generation being directly proportional to tau concentration under these experimental conditions.
Figure 2.3. Critical concentration for tau filament and intermediate formation in the presence of anionic microspheres.

Carboxylate-substituted polystyrene microspheres (90 nm diameter, 11.6 Å²/eq molecular area; 124 pM) were incubated (24 h at 37°C) with varying concentrations of htau40 (0 - 4 µM) in the presence of ThS (4 µM) and then assayed for fibrillization by EM (○) and intermediate formation by ThS fluorescence (●) assays. Each data point represents the mean ± SD of triplicate determinations whereas the solid lines represent best fit of the data points to a linear regression (equation 2). In the case of fibrillization, the regression line intercepted the abscissa at 2.06 ± 0.10 µM htau40, indicating the existence of a critical concentration. In contrast, ThS fluorescence was detectable below the critical concentration of fibrillation, with the regression line intersecting the abscissa at the origin. Whereas fibrillization was characterized by a critical concentration, appearance of ThS fluorescence was not.
Figure 2.4. Intermediate formation is accompanied by changes in secondary structure.

Htau40 (2.0 µM) was incubated (20 min at 25°C) in 100 mM sodium perchlorate, 20 mM boric acid, pH 7.4 in the presence (●) or absence (○) of 50 µM AA inducer, then immediately subjected to far-UV CD spectroscopy (37°C). (A) Intermediate formation was accompanied by an increase in [θ]_{195} and a decrease in [θ]_{220}, consistent with the formation of secondary structure. (B) CD spectra were individually fit by CDSTTR, SELCON3, and CONTIN/LL algorithms to calculate estimates of total α-helical, β-strand, and random coil/turn conformation. The three estimates were then averaged and plotted ± SD for htau40 in the presence (black bars) or absence (white bars) of AA inducer. Intermediate formation was accompanied by increases in β-conformation and decreases in random coil/turn conformation. *, p < 0.05, comparing the two populations.
Figure 2.5. Effects of intermediate formation on ANS fluorescence spectra.

Reactions containing assembly buffer and no additions (●), 1.9 µM htau40 (■), 1.9 µM htau40 and 50 µM AA inducer (□), or 0.8 µM protamine and 50 µM AA inducer (○) were prepared. After incubation (24 h at 25°C), aliquots were removed, treated with 20 µM ANS (final concentration), and immediately subjected fluorescence spectroscopy ($\lambda_{ex} = 350$ nm; $\lambda_{em} = 400 – 600$ nm). ANS in buffer alone fluoresced weakly at optimum wavelength of $\lambda_{em} = 500$ nm (●). In contrast, intermediates prepared by incubation of htau40 with AA inducer fluoresced brightly with a blue-shifted optimum of $\lambda_{em} = 487$ nm (□). Neither tau without inducer (■) nor AA micelles formed in the presence of protamine (○) were capable of recapitulating the ANS fluorescence enhancement observed in the presence of htau40 with AA inducer, indicating that ANS binds preferentially to tau intermediates.
Figure 2.6. Planar aromatic dyes trigger tau fibrillization.

Htau40 (4 µM) was incubated (24 h at 37°C) under standard conditions with (A), 25 µM Congo Red (CR); (B), 100 µM Thiazin red (TR); (C), 100 µM ThS; (D), 75µM AA (3h, 37°C); or (E), 100 µM ANS. Planar aromatic dyes with known ability to bind β-sheet structure such as Congo Red, Thiazin red and ThS induced the formation of tau filaments with twisted ribbon morphology (see Figure 2.9 for higher magnification image) clearly distinct from the straight filaments induced by AA. In contrast, the aromatic dye ANS, which binds hydrophobic clusters in the partially folded intermediate, did not have inducer activity when tested in the range 5 – 500 µM. These data indicate that dyes complementary to β-sheet structure induce tau fibrillation, and that the filament morphology observed reflects the fibrillization inducer employed. Bar = 100 nm.
Figure 2.7. Potency of thiazin red dependent tau fibrillization.

The dependence of tau fibrillization equilibrium on thiazin red concentration (0 - 1000 µM) was determined in the presence of 1 (○) or 2 µM (●) htau40 after 24 h incubation at 37°C by quantitative electron microscopy. Each data point represents the mean ± SD of triplicate determinations whereas the solid lines represent best fit to a log-normal distribution (equation 3). The concentration response curves were biphasic, with an optimum centered on ~114 µM.
Figure 2.8. Thiazin red modulates tau nucleation rate.

The time course (37°C) of htau 40 (0.8 µM) fibrillization in the presence of 50 (○) or 100 (●) µM thiazin red was determined by EM. Each data point represents total filament length/field (Γf) as a function of time whereas each sigmoid curve represents the best fit of the data points to a three parameter Gompertz function. Lag times calculated as described in Experimental Procedures were 0.73 ± 0.09 h and 0.30 ± 0.04 h for 50 and 100 µM thiazin red, respectively. The concentration dependence of lag time was consistent with thiazin red increasing the rate of tau filament nucleation.
Carboxylate-substituted polystyrene microspheres (90 nm diameter, 11.6 Å²/eq molecular area; 124 pM) were incubated (24 h at 37°C) with htau40 (4 µM) in the (A), absence or (B), presence of 50 µM thiazin red then visualized by transmission EM. In the absence of thiazin red, microspheres induced the formation of straight filaments, with frequency rarely exceeding one nucleation event per bead. In contrast, the presence of thiazin red increased nucleation frequency so that multiple filaments per bead were a common occurrence. Note the change in filament morphology to twisted ribbon in the presence of thiazin red. (C), statistical distributions of the number of nucleation events per bead were then calculated for reactions conducted in the presence of 0, 50, and 100 µM thiazin red (TR). Each bar represents the number of nucleations (filaments) per bead normalized as percent frequency, whereas each solid line represents best fit of the data to the Poisson distribution (equation 4). Thiazin red increased nucleation frequency so that more multiple filaments per bead were observed. Bar = 100 nm.
Figure 2.10. Critical concentration of tau fibrillization in the presence of thiazin red.

The dependence of fibrillization equilibrium on tau concentration (0.1-2 µM) was determined in the presence of 50 (○) or 100 µM (●) thiazin red after 24 h incubation at 37°C by quantitative electron microscopy. Each data point represents the mean ± SD of triplicate determinations whereas the solid lines represent best fit to a linear regression. Critical concentrations determined from the intercepts of regression lines with the abscissa were 0.20 ± 0.07 and 0.21 ± 0.05 at 50 and 100 µM thiazin red concentrations, respectively.
Figure 2.11. Thiazin red induces tau fibrillization in monomeric form.

Thiazin red was dissolved in either methanol (●), assembly buffer (○), or assembly buffer containing 4 µM htau40 (■), incubated 24 h at 37°C, and finally subjected to absorbance scans in the range of 400 – 600 nm. All curves showed an absorbance maximum centered on 514 nm with a shoulder at 548 nm, suggesting that thiazin red did not aggregate into supermolecular complexes under standard assay conditions.
CHAPTER 3

3 C-TERMINAL TRUNCATION MODULATES BOTH NUCLEATION AND EXTENSION PHASES OF TAU FIBRILLIZATION

3.1 Introduction.

Proteolytic post-translational modification of various proteins contributes to the pathogenesis of AD (Selkoe, 2004), Huntington’s disease (Chung et al., 2001), and gelsolin amyloidosis, Finnish type (Gamblin et al., 2003) by driving formation of fragments with increased amyloidogenic potential relative to their full-length precursor forms. Proteolytic modification also has been implicated in the formation of toxic and aggregation-prone fragments of tau protein, a microtubule-associated protein that aggregates into filaments within the characteristic neuritic lesions of frontotemporal dementias as well as AD (Guillozet-Bongaarts et al., 2005). Tau truncations can be detected at early stages of AD cognitive decline (Abraha et al., 2000) and their accumulation in AD hippocampus correlates inversely with cognition (Rissman et al., 2004). Tau sequences C-terminal to the microtubule-binding region are particularly sensitive to proteolysis, and are substrates for cathepsins (Kenessey et al., 1997), caspases (Gamblin et al., 2003; Guo et al., 2004; Kang et al., 2005; Rissman et al., 2004; Rohn et al., 2002), and other proteases. Caspase-mediated cleavage after Asp$^{421}$ (using the
residue numbering of full-length wild-type four repeat tau isoform htau40; (Bramblett et al., 1993)) can be detected in mild cognitive impairment and AD but not in non-demented control brains (Gamblin et al., 2003; Rissman et al., 2004). As neuritic pathology progresses, C-terminal truncations extend beyond Asp\(^{421}\) to at least as far as Glu\(^{391}\), which lies adjacent to the C-terminal limit of the protease-resistant core of brain-derived paired helical filaments (Novak et al., 1993; Wischik et al., 1988). Relative to full-length htau40, purified recombinant tau proteins truncated after Glu\(^{391}\) or Asp\(^{421}\) (i.e., htau40\(^{A392}\) and htau40\(^{A422}\)) are prone to aggregation \textit{in vitro}, yielding faster rates of fibrillization and more filament mass at reaction plateau (Abraha et al., 2000; Rissman et al., 2004). C-terminally truncated tau also has been suggested to “seed” aggregation reactions through secondary nucleation, where pre-formed filaments composed of truncated tau serve as the nidus for addition of full-length tau molecules (Cotman et al., 2005; Rissman et al., 2004). Nonetheless, neither the mechanism through which these effects occur nor its significance for lesion formation are known.

Clarifying how C-terminal truncation modulates tau aggregation has been difficult in part because full-length tau protein fibrillizes poorly \textit{in vitro} in the absence of exogenous inducers (Abraha et al., 2000). These agents, which include anionic surfactant micelles and anionic microspheres, promote heterogeneous nucleation (\textit{i.e.}, nucleation owing to the presence of foreign particles) on their surfaces from which tau filaments then extend in an elongation reaction (Chirita et al., 2005; Chirita and Kuret, 2004; Chirita et al., 2003). Heterogeneous nucleation may mediate the aggregation-inducing activity of other polyanionic macromolecular inducers as well, such as
glycosaminoglycans (Hasegawa et al., 1997; Perez et al., 2002). Because the rate of heterogeneous nucleation depends on the concentration of nucleating particles (or their surface area) in addition to protein supersaturation (Huang et al., 2001), the effects of post-translational modifications on tau aggregation have been difficult to interpret mechanistically. Moreover, the use of static light scattering methods uncorrected for the presence of inducer to assess the effect of tau C-terminal truncation on reaction rates (Abraha et al., 2000; Rissman et al., 2004) has complicated interpretation of reaction progress data (Necula and Kuret, 2004c).

Here we examine the effect of C-terminal truncation in the presence of thiazin red, a small-molecule fibrillization inducer of full-length tau isoforms. Small-molecule dyes appear to drive aggregation reactions by stabilizing assembly-competent protein conformations (Chirita et al., 2005; Chung et al., 2001). Once these conformations are adopted, aggregation proceeds spontaneously much like reactions mediated by homogeneous nucleation. Furthermore, the resultant amyloidogenic conformations support fibrillization at submicromolar tau concentrations, well within levels thought to be physiological in vivo (Chirita et al., 2005; Chirita and Kuret, 2004). Under these conditions, it is possible to detect and quantify the effect of post-translational modification on both the nucleation and elongation phases of the fibrillization reaction. The results indicate that C-terminal truncation accelerates tau fibrillization principally by lowering critical concentration but also by directly accelerating the nucleation reaction.
3.2 Materials and methods.

Materials. Recombinant His-tagged wild-type htau40 and C-terminal truncation mutants htau40\(^{\Delta392}\) and htau40\(^{\Delta422}\) were prepared as described previously (Abraha et al., 2000; Carmel et al., 1996). Stock solutions of fibrillization inducer thiazin red were prepared as described (Chirita et al., 2005).

Tau aggregation assay. Tau preparations were incubated without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) at room temperature for up to 24 h in the presence or absence of 100 \(\mu\)M thiazin red inducer. For TEM analysis, aliquots were removed, treated with 2% glutaraldehyde (final concentration), mounted on formvar/carbon-coated 300 mesh grids, and negatively stained with 2% uranyl acetate as described previously (Chirita et al., 2003; King et al., 1999). The lengths of all filaments were estimated using ImageJ software (National Institutes of Health, Bethesda, MD) without a length cutoff filter.

Analytical methods. Sigmoidal reaction progress curves were fit to the Gompertz function (Necula and Kuret, 2004c):

\[
y = ae^{-e^{\left(\frac{t-t_i}{b}\right)}}
\]

where \(y\) is total filament length measured by TEM at time \(t\), \(t_i\) is the inflection point corresponding to the time of maximum growth rate, \(a\) is the maximum total filament length at plateau, and \(b = 1/k_{app}\), where \(k_{app}\) is the apparent first order rate constant describing growth of the filament population in units of time\(^{-1}\). Lag times, defined as the
time where the tangent to the point of maximum polymerization rate intersects the abscissa of the sigmoidal curve (Evans et al., 1995), were calculated as $t_i - b$ (Necula and Kuret, 2004c).

Critical concentrations were determined by incubating tau samples (0.2 – 1.0 µM, bulk concentrations) for 24 h in assembly buffer at room temperature in the presence of 100 µM thiazin red without agitation. Samples were analyzed by transmission electron microscopy as described above, and critical concentrations were estimated from the abscissa intercepts of concentration response curves after least squares linear regression. All statistical parameters estimated from linear (e.g., critical concentration) or non-linear (e.g., lag time) regression analyses are presented as ± S.E. of the estimate.

3.3 Results.

3.3.1 C-terminal truncation modulates critical concentration

C-terminal truncation yields more filament mass than wild-type htau40 when incubated in the presence of anionic inducer under reducing conditions (Abraha et al., 2000; Rissman et al., 2004). To determine whether this effect could be reproduced in the presence of thiazin red inducer, equal bulk concentrations of wild-type htau40 and truncation mutants htau40$^{A392}$ and htau40$^{A422}$ were incubated at room temperature in the presence of 100 µM thiazin red for 24 h and examined by TEM. These two mutants were selected for study because each represents a histochemically confirmed cleavage event,
with htau40^{\Delta422} corresponding to caspase-mediated proteolysis detectable in early stage AD and htau40^{\Delta392} corresponding to near maximal C-terminal cleavage (Gamblin et al., 2003; Novak et al., 1993). All preparations fibrillized with twisted morphology as described previously (Chirita et al., 2005), with no morphological differences apparent among samples (Fig. 3.1). However, both htau40^{\Delta392} and htau40^{\Delta422} truncation mutants yielded more and longer filaments compared to wild-type htau40 (Fig. 3.1). These data indicate that the aggregation promoting effects of C-terminal truncation are inherent in the preparations and not dependent on members of the polyanionic family of inducers such as glycosaminoglycans (Rissman et al., 2004) or anionic-surfactant micelles (Abraha et al., 2000).

The amount of tau filament mass attained after the prolonged incubation described above corresponds to the plateau phase of tau fibrillization when the rate of protein addition to filament ends equals the rate of protomer dissociation (evidence for plateau phase is shown in Section 3.2 below). That equilibrium is characterized by a “critical” concentration corresponding to the maximum solubility of protein above which all additional assembly-competent protein enters the polymeric phase (Harper and Lansbury, 1997). It is the highest concentration of protein incapable of supporting fibrillization, and therefore can be estimated from the dependence of fibrillization on bulk protein concentration (Timasheff, 1981). To determine whether C-terminal truncation modulated this equilibrium, the critical concentration of htau40^{\Delta392} and htau40^{\Delta422} were estimated after 24 h incubation at room temperature using TEM and compared to values for wild-type htau40. The value for wild-type htau40 in the presence of thiazin red
inducer was $207 \pm 9$ nM (Fig. 3.2B; Table 3.1). In contrast, critical concentrations progressively decreased with truncation to values as low as $60 \pm 29$ nM (Table 3.1). These data suggest that the increased fibrillization at reaction plateau produced by C-terminal truncation resulted from higher affinity between filament ends and assembly competent tau.

### 3.3.2 C-terminal truncations modulate nucleation rates.

Small molecule dyes such as thiazin red appear to induce protein aggregation with near homogeneous nucleation kinetics because they act to stabilize partially folded, aggregation prone conformations in solution (Chirita et al., 2005; Chung et al., 2001). Under these conditions, the degree of protein supersaturation provides the principal driving force for nucleation (Kashchiev, 2000). As a result, the rate of homogeneous nucleation is directly dependent on the degree of supersaturation (Fesce et al., 1992):

$$\frac{dF}{dt} = K_n (A - K_{crit})^n$$

where $dF/dt$ is the rate of formation of filament nuclei, $A-K_{crit}$ is the concentration of monomer above the critical concentration ($i.e.,$ the degree of supersaturation), $n$ represents the nucleus cluster size, and $K_n$ is an operational quantity representing the homogeneous nucleation rate constant with units that depend on the size of $n$. Therefore, post-translational modifications that lower the critical concentration, increase $K_n$, or increase nucleus cluster size $n$ will increase the rate of filament nucleation at a given bulk tau concentration. To assess the influence of C-terminal truncation on nucleation rate
independent of supersaturation effects, the fibrillization time courses of htau40, htau40\textsuperscript{\Delta392} and htau40\textsuperscript{\Delta422} were followed by TEM over a period of 24 h (room temperature, no agitation) at constant supersaturation (i.e., bulk tau concentrations were varied on the basis of measured $K_{\text{crit}}$ summarized in Table 3.1 so that $A-K_{\text{crit}}$ for all reactions was 600 nM). All resultant reaction progress curves were sigmoidal with clear lag, exponential growth, and plateau phases (Fig. 3.3). Despite being incubated at lower bulk tau concentrations, and equivalent levels of supersaturation, both C-terminal truncations aggregated with significantly shorter lag times than did wild-type htau40 (Fig. 3.3; Table 3.1). Because lag times vary inversely with nucleation rate, these data indicate that truncation of as little as 20 residues from the C-terminus of tau can accelerate nucleation rate. As a result, the pseudo-first order rate constant for aggregation, $k_{\text{app}}$, approximately doubled with C-terminal truncation when supersaturation was held constant (Table 3.1). These data indicate that C-terminal truncations are capable of modulating both pre- and post-nuclear equilibria, leading to lower critical concentrations and faster rates of nucleation. Both of these effects contribute to the increased velocity of aggregation observed macroscopically and underlie the amyloid-promoting effects of truncation.

3.4 Discussion.

Tau filament formation in tauopathic neurodegenerative diseases such as AD appears to follow a primary nucleation mechanism: the phase transition proceeds from non-fibrillar tau without the participation of preexisting filaments formed as a result of
normal cell function. Early morphological analyses of neurofibrillary lesions completed using TEM found tau filaments accumulating within the neuronal cytoplasm (Kidd, 1964). These appeared to enter and exit the plane of section owing to the curvature of the cell and therefore gave the appearance of filament ends being free in the cytoplasm. This morphological pattern is superficially consistent with primary nucleation being homogeneous, where tau molecules spontaneously self-associate to nucleate and extend filaments without the contribution of foreign particles. The results found herein suggest that under these aggregation conditions, C-terminal truncation would have a significant effect on both the nucleation and extension phases of fibrillization. Nucleation would be greatly accelerated, owing to the decrease in critical concentration, which increases fibrillization driving force, and to the direct and positive effect of truncation on nucleation efficiency. The decrease in critical concentration also would better support fibrillization at the low free tau concentrations that likely exist at early stages of the fibrillization reaction. Filaments formed from preferential nucleation and extension of C-terminally truncated tau may facilitate secondary nucleation of full-length monomeric tau and therefore mediate the recently postulated “seeding” effect of truncated tau \textit{in vitro} (Cotman et al., 2005; Rissman et al., 2004).

More recent morphological analyses of neurofibrillary tangles employed serial sections to follow the course of filaments through the neuronal cytoplasm (Gray et al., 1987). These studies found that many filament ends are in fact not free in solution, but intimately associated with intracellular bodies (Gray et al., 1987). Moreover, at least a portion of tau molecules begin the transition from natively unfolded to extended β-sheet
conformation in association with intracellular membranes (Galvan et al., 2001). Together these observations suggest that heterogeneous nucleation and growth of tau filaments may be the dominant pathway \textit{in vivo}, where foreign bodies supply a surface which selectively binds tau and facilitates the adoption of extended \(\beta\)-sheet structure and filament nucleation at levels of supersaturation well below those needed to support homogeneous nucleation (Chirita and Kuret, 2004). Rates of heterogeneous nucleation depend almost entirely on inducer concentration as supersaturation rises (Huang et al., 2001). Under these conditions, the nucleation-promoting effects of C-terminal truncation may be substantially lower than under homogeneous nucleation conditions. For example, certain tau pseudophosphorylation mutants have lower \(K_{\text{crit}}\) than wild-type tau and therefore greater supersaturation at a given bulk tau concentration, but this does not lead to faster nucleation rates under heterogeneous nucleation conditions (Necula and Kuret, 2004b). Similarly, the primary amyloidogenic mechanism for C-terminal truncations that lower \(K_{\text{crit}}\) under heterogeneous nucleation conditions would be to support filament formation at low free tau concentrations (\textit{i.e.}, when tau is mostly bound by microtubules).

Finally, recent studies suggest that C-terminal truncation, while occurring early in the course of disease, actually occurs after filament nucleation (Guillozet-Bongaarts et al., 2005). Under these conditions, truncation would have no role in modulating nucleation, but could facilitate the accumulation of filaments at early stages of disease when ambient free tau concentrations are low. Thus, even when occurring late in the aggregation pathway, C-terminal truncation is predicted to have significant positive effects on aggregation efficiency.
In each of the scenarios summarized above, C-terminal truncation does not necessarily trigger the conformational changes needed for aggregation to occur, but rather enhances the reaction by lowering critical concentration and making nucleation more efficient. The effect is somewhat surprising because the tau molecule C-terminal to the microtubule-binding region is acidic, and so virtually any truncation leads to a modest increase in both isoelectric point and net charge at physiological pH. On the basis of electrostatic repulsion, both of these changes should raise solubility and decrease rates of aggregation (Chiti et al., 2003). In the case of tau protein, however, it appears that other factors (such as specific charge-charge interactions, surface exposure of hydrophobic residues, or stabilization of extended conformation; (Calamai et al., 2003; Chiti et al., 1999; Gazit, 2002; Schmittschmitt and Scholtz, 2003)) rather than isoelectric point predominate under the near-physiological assembly conditions employed here. For example, the aggregation promoting effect of tau pseudophosphorylation mutagenesis is site specific, even though isoelectric point decreases equally in all mutations (Necula and Kuret, 2004b; Necula and Kuret, 2005). Moreover, the depression of $K_{\text{crit}}$ observed in pseudophosphorylation mutant htau40$^{T212E}$ relative to wild-type htau40 results from diminished rates of disaggregation rather than increased rates of aggregation (Necula and Kuret, 2005). These results parallel the effect of surface salt-bridge formation on globular protein folding stability, which acts to stabilize the folded state by decreasing rates of unfolding without increasing rates of folding (Makhatadze et al., 2003; Makhatadze et al., 2004).
In summary, C-terminal truncation of as few as 20 residues from full-length four-repeat tau can have significant effects on aggregation kinetics by acting at the levels of filament nucleation and extension. The specific effects on neuritic lesion formation will depend on the timing of truncation relative to filament nucleation in disease. However, the extension promoting activity is especially pronounced and will act to support tau fibrillization at low free tau concentrations under all pathophysiologically relevant scenarios.

3.5 Summary.

Proteolytic post-translational modification has been proposed as an early stage event in the aggregation of tau protein and formation of neurofibrillary lesions in Alzheimer’s disease. Caspases and other proteases cleave tau in vivo at discrete locations including Asp\textsuperscript{421} and Glu\textsuperscript{391}. Both cleavage products are prone to aggregation relative to wild-type, full-length tau protein. To determine the mechanism underlying this effect, the fibrillization of tau truncated after Asp\textsuperscript{421} and Glu\textsuperscript{391} residues was characterized in a full-length four-repeat tau background using quantitative electron microscopy methods under homogeneous nucleation conditions. Both C-terminal truncations decreased critical concentration relative to full-length tau, resulting in more filament mass at reaction plateau. Moreover, truncation directly augmented the efficiency of the nucleation reaction. The results suggest the mechanism through which C-terminal proteolysis can modulate tau filament accumulation depending on whether it precedes or follows nucleation.
3.6 Tables.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{\text{crit}}$ (nM)</th>
<th>$^a$Lag time (h)</th>
<th>$^a$k$_{\text{app}}$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Htau40</td>
<td>207 ± 9</td>
<td>1.84 ± 0.12</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>Htau40$^{A422}$</td>
<td>156 ± 13</td>
<td>1.16 ± 0.07</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>Htau40$^{A392}$</td>
<td>60 ± 29</td>
<td>1.13 ± 0.12</td>
<td>0.45 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$Determined at constant supersaturation ($A - K_{\text{crit}} = 600$ nM).

Table 3.1. Kinetic constants for tau aggregation reactions.
3.7 Figures.

Figure 3.1. C-terminal truncation modulates tau fibrillization.

Fibrillization of htau40 and C-terminal truncation mutants htau40Δ422 and htau40Δ392 (all at 600 nM bulk concentration) was viewed by TEM after 24 h incubation at 20°C in the presence of thiazin red inducer (100 µM). C-terminal truncation resulted in increased numbers and total length of filaments.
Figure 3.2. C-terminal truncation modulates critical concentration.

The dependence of fibrillization plateau on tau concentration was determined in the presence of 100 µM thiazin red after 24 h incubation at 20°C by quantitative electron microscopy. Each data point represents the mean ± SD of triplicate determinations (htau40, ●; htau40Δ422, ○; htau40Δ392, ■) whereas the solid lines represent best fit of the data points to a linear regression. The critical monomer concentration, which was estimated from the abscissa intercept of each regression line (summarized in Table 3.1), was modulated by C-terminal truncation.
Figure 3.3. C-terminal truncation speeds the rate of tau aggregation.

The time course (20°C) of htau 40 (0.81 μM; ●), htau40∆422 (0.76 μM; ○), and htau40∆392 (0.67 μM; ■) fibrillization in the presence of 100 μM thiazin red inducer was determined by TEM. Protein concentrations were varied so that the driving force for nucleation (A-K_{crit}) of all samples was held constant at 0.6 μM. Each data point represents the average of duplicate determinations of total filament length/field (Γf, expressed as a percentage of the value at plateau) whereas each sigmoid curve represents the best fit of the data points to a three-parameter Gompertz function (equation 1). Even when adjusted for differences in critical concentration, C-terminal truncation mutants aggregated with shorter lag times than full-length htau40, suggesting that C-terminal truncation increases nucleation efficiency.
CHAPTER 4

4 TAU AGGREGATION AND TOXICITY IN A CELL CULTURE MODEL OF TAUOPATHY

4.1 Introduction.

Tau is a microtubule-binding protein expressed from a single gene on human chromosome 17 (Buee et al., 2000). In Alzheimer’s disease (AD), full-length tau isoforms aggregate in neuronal cell bodies to form neurofibrillary tangles and in neuronal processes to form neuropil threads and dystrophic neurites associated with neuritic plaques (Buee et al., 2000). Tau aggregates appear as filaments, within which a portion of each tau molecule adopts β-sheet conformation. As in other filamentous aggregates, the resultant β-sheets stack in parallel, with each sheet orthogonal to the axis of the growing tau filament (Margittai and Langen, 2004). Neurofibrillary lesion formation is accompanied by 4 – 8 fold increases in total brain tau levels (Khatoon et al., 1992; Khatoon et al., 1994). Because expression of total tau does not change at the mRNA level in AD (Hyman et al., 2005; Yasojima et al., 1999), disease-associated increases in tau protein appear to derive from post-translational events (e.g., fibrillization) that decrease tau turnover. Tau accumulation in AD may result from the resistance of filamentous tau to proteasome-mediated degradation (Keck et al., 2003).
Tau fibrillization correlates spatially and temporally with neurodegeneration and cognitive decline (Arriagada et al., 1992; Ghoshal et al., 2002). As a result, tau lesions densities are used for postmortem staging and diagnosis of AD (Braak and Braak, 1991). These correlations, along with the discovery that mutations in the tau gene cause familial forms of frontotemporal dementia (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998), suggest that tau misfunction is somehow associated with neurodegeneration. Three general hypotheses have been put forward to rationalize a direct relationship between tau fibrillization and disease. The first argues that tau aggregates are directly toxic to cells that harbor them. For example, tau aggregates are capable of inhibiting the ubiquitin-proteasome system (Keck et al., 2003). Inhibition of this system, which plays important roles in cell homeostasis, has been proposed to be a source of cellular stress (Bence et al., 2001; Bennett et al., 2005). The second argues that sequestration of tau from microtubules destabilizes the cytoskeleton leading to a loss-of-function toxicity (Alonso et al., 1996). Although loss of tau is well tolerated over both long (Harada et al., 1994; Ikegami et al., 2000) and short (Tint et al., 1998) time periods, simultaneous loss of both tau and other microtubule associated proteins may lead to more severe phenotypes (DiTella et al., 1996). A third hypothesis is that tau modified post-translationally (e.g., by hyperphosphorylation or truncation) may be directly toxic to cells (Park and Ferreira, 2005; Shimura et al., 2004). Under these conditions, aggregation could be protective for cells by sequestering toxic species.

Resolving the role of tau fibrillization is important for assessing the potential of tau-directed therapies. However, directly testing the above hypotheses in biological
models has been complicated by the resistance of full-length tau isoforms to spontaneous aggregation over experimentally tractable time periods (Chirita et al., 2005). The problem has been overcome by high level tau overexpression alone (Andorfer et al., 2005; Wittmann et al., 2001) or in combination with aggregation promoting mutations (Santacruz et al., 2005). Conditions that drive aggregation-promoting post-translational modifications (e.g., phosphorylation; Sato et al., 2002) also have been successful. But mere overexpression of normal tau in neurons results in a toxic phenotype (Terwel et al., 2005). Because normal tau protein levels do not increase in AD (Khatoon et al., 1994), the significance of overexpression-mediated toxicity found in transgenic systems has been ambiguous.

Recently we found that the kinetic barrier to full-length tau fibrillization can be overcome by small-molecule ligands that bind β-sheet structure such as Congo red, thiazin red, and thioflavin S (Chirita et al., 2005). The ability of Congo red to populate amyloidgenic protein conformations (Chung et al., 2001) suggests that these agents drive aggregation by thermodynamic linkage of their binding reaction with the tau self-association reaction (linkage refers to the influence of ligand binding upon other equilibria including aggregation reactions and phase transitions; Wyman and Gill, 1990). As a result, fibrillization agonists allow the study of full-length tau fibrillization in the absence of post-translational modifications, mutations, or exogenous anionic inducers such as heparin (Chirita et al., 2005).
Here we extend this approach to cell culture as a means of examining the role of full-length tau fibrillization on acute toxicity. The results suggest that large scale aggregation in HEK-293 cells is well tolerated, but that a growth impaired phenotype is unmasked in the presence proteasome inhibitor ALLN.

4.2 Materials and methods.

Materials. Monoclonal antibodies Tau5, Tau12, and PHF1 were obtained from Dr. L. I. Binder, Northwestern University Medical School, Dr. Peter Davies, Einstein College of Medicine, respectively. Monoclonal anti-α-tubulin antibody DM-1A (Blose et al., 1984) and protease inhibitors (1x complete contained: 1 mM 4-(2-aminoethyl)-bezenesulfonylfluoride, 0.8 µM aprotinin, 40 µM bestatin, 20 µM leupeptin, 15 µM pepstatin A, 14 µM L-transepoxysuccinyl-leucylamido-[4-guanidino]butane, and 26 µM ALLN) were obtained from Sigma/Aldrich Chemicals (St. Louis, MO). HEK-293 cells were obtained from ATCC (Manassas, VA), whereas a HEK-293 derived cell line stably expressing human htau40 (tau cells) was prepared as described previously (Ko et al., 2004). Purified bovine brain tubulin was from Cytoskeleton (Denver, CO). CR (City Chemical LLC, West Haven, CT) was stored as a 50 mM stock solution in DMSO. Recombinant htau40 containing polyhistidine tag (His$_6$-htau40) was expressed and purified as described (Carmel et al., 1996).

In vitro tau assembly. His$_6$-htau40 (1 µM) was incubated with various concentration of CR for varying time periods in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM DTT) at 37°C as described previously (Chirita et al., 2003; King et al., 1999). Aliquots were removed and assayed for solubility by centrifugation (described below) or
for fibrillization by electron microscopy. For the latter assay, samples were mounted on Formvar/carbon-coated 300 mesh grids for 2 min, then stained with 2% uranyl acetate for 1 min (Chirita et al., 2003; King et al., 1999). Images were viewed in a Phillips CM 12 transmission electron microscope operated at 65 kV, and captured on film at 22,000-fold magnification. Five fields were imaged per sample. All filaments in each image were counted and average total length adsorbed per field ($\Gamma_i$) was calculated ± SD.

**Cell culture and treatment.** HEK-293 and tau cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% bovine serum, 100-units/ml penicillin G, 250 ng/ml amphotericin B, and 100 µg/ml streptomycin (37°C with 5% CO₂). Tau cells were maintained in selective medium containing 500 µg/ml G418. To assay the effect of CR on tau aggregation and cell viability, all cells were plated and cultured for 24 h, treated with different concentrations of CR, and harvested after 1 - 14 days of treatment. Media, including CR, was refreshed every 3 days.

**Isolation of soluble and insoluble tau.** The solubility of cell-derived tau protein was assayed under microtubule-destabilizing conditions. CR-treated tau cells were suspended in lysis buffer (20 mM Tris-HCl pH 7.4, 140 mM NaCl, 1 mM PMSF, 1 mM Na₃NO₄, 10 mM NaF, 0.5% v/v NP-40, 1 mM EDTA, and 1x complete protease inhibitors), subjected to three freeze/thaw cycles (Vogelsberg-Ragaglia et al., 2000), and then incubated at 4°C for 30 min to induce the disassembly of microtubules. Nuclei were removed from the lysates by centrifugation at (1000g for 5 min) at 4°C.

The resultant extracts were centrifuged (100,000g for 1 h) at 4°C. The resultant pellets were washed three times with lysis buffer, then resuspended in lysis buffer (1/3
v/v relative to the total volume of supernatant). Equal volumes of pellet and supernatant fractions were boiled and subjected to immunoblot analysis after SDS-PAGE electrophoresis (10% polyacrylamide) as described below.

Aliquots of *in vitro* aggregation reactions were processed similarly after centrifugation, except that pellets were washed with PBS before being resuspended into SDS-sample buffer.

**Immunoelectronmicroscopy.** To assay morphology, tau aggregates were isolated from CR-treated tau cells by a modification of the procedure used to isolate authentic PHF (Greenberg and Davies, 1990). Cells were homogenized in ice cold buffer H (10 mM Tris-HCl, 1 mM EGTA, 0.8 M NaCl, 10% sucrose and protease inhibitor cocktail, pH 7.4), and centrifuged for 20 min at 20,000 g. The supernatant (S1) was collected and the pellet (P1) was re-homogenized in buffer H, and separated into S2 and P2 fractions by centrifugation (20 min at 20,000 g). Supernatant fractions S1 and S2 were combined and incubated with 1% Sarkosyl for 1 h at room temperature with agitation. After centrifugation of the mixture (1 h at 100,000 g), the resultant pellets were resuspended in 50 mM Tris-HCl (pH 7.4). Aliquots were absorbed to Formvar/carbon-coated grids and incubated with the Tau-12 monoclonal antibody for 30 min at 37°C. After washing with 0.1% gelatin, the grids were incubated with the 10-nm gold labeled goat anti-mouse IgG + IgM (Amersham Bioscience). Finally, the samples were stained with 2% uranyl acetate for 1 min, and then analyzed in a Phillips CM 12 transmission electron microscope as described above.
**Tubulin binding assays.** To assess tubulin binding activity of tau aggregates prepared *in vitro*, His$_6$-htau40 (1 µM) treated with CR (10 µM) for various lengths of time was incubated with 1 mg/ml purified tubulin at 37°C for 30 min in binding buffer (80 mM PIPES, pH 6.8, 1 mM MgCl$_2$, 1 mM EGTA, 1 mM GTP) in a final volume of 100 µl. Reactions were incubated with 1 µg monoclonal anti-α-tubulin antibody for 2 h after which time protein-G-agarose beads (40 µl of 25% (w/v) slurry) were added and incubation continued for 1 h. Immunoprecipitates were collected by centrifugation (2000g for 5 min), washed three times with lysis buffer, and finally subjected to immunoblot analysis as described below. For these experiments, input tubulin was visualized by Coomassie blue staining after SDS-PAGE.

To assay the influence of aggregation on the ability of tau to bind endogenous tubulin, lysates (100 µg) were prepared from CR-treated or control tau cells as described above, and then tubulin was immunoprecipitated with anti-α-tubulin antibody and protein-G-agarose as described above. For these experiments, levels of endogenous tubulin cell lysates were estimated by immunoblot analysis with anti-α-tubulin antibody.

**Immunoblot analysis.** Samples were boiled in SDS-PAGE loading buffer, subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes as described previously (Ko et al., 2004). Membranes were sequentially blocked with blocking buffer (Ko et al., 2004) containing 5% milk for 1 h, primary antibody for 2 h at room temperature, and secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG) for 1h. The protein bands were visualized by enhanced chemiluminescence and quantified with a Bio-Rad GS-800 calibrated laser densitometer.
**Immunocytochemistry.** Wild type HEK-293 cells and tau cells were cultured on glass coverslips for 24 h and treated with 10 µM CR for up to 7 days. Cells were chilled on ice for 30 min, washed 3-times with PBS 3, and then fixed with methanol. Following PBS washes, cells were sequentially incubated with PBS containing 3% bovine serum albumin for 1 h, Tau5 antibody (1:1000) overnight at 4°C, and fluorescein-labeled goat anti-mouse IgG secondary antibody (1:1000) for 1 h. The coverslips were mounted with 50% glycerol. Immunostaining was visualized with an MRC 1024 krypton/argon laser-scanning confocal system (Bio-Rad) equipped with a Nikon Optiphot II photomicroscope (Melville, NY) and operated with a FITC-HYQ filter set (λ_em = 535/50) at λ_ex = 488 nm.

**Cell viability assay.** HEK-293 and tau cells were cultured on coverslips for 24 h, then treated with vehicle (DMSO) or various concentration of CR for up to 14 days. Cells were then washed three times with PBS to remove the dead cells, collected by trypsinization, and finally counted in triplicate with a hemacytometer.

To determine the effect of ALLN on cell viability, tau cells were plated on coverslips as described above and then treated with DMSO vehicle or 10 µM CR for 4 days. ALLN (26 µM) or ethanol was added to the medium in the presence or absence of CR for another 3 days. The cells were harvested and counted as above.

**Analytical methods.** Aggregation progress curves were modeled as a monomolecular growth scheme assuming pseudo-first order kinetics. Filament growth was fit to the function:

\[
y = y_0 + y_{\infty} (1 - e^{-k_{app}t})
\]

Eq (1)
where \( y, y_0, \) and \( y_\infty \) correspond to filament length or mass at time \( t \), at time zero, and at infinite time, respectively, and \( k_{\text{app}} \) is the apparent first order rate constant. For \textit{in vitro} reactions, \( y_0 \) was always set equal to zero.

Consistent with this model, time-dependent loss of soluble tau was fit to a simple exponential decay function:

\[
m = m_0 e^{(-k_{\text{app}} t)}
\]

where \( m \) and \( m_0 \) correspond to soluble tau mass at time \( t \) and time zero, respectively, and \( k_{\text{app}} \) is the apparent first order decay rate constant.

Concentration response curves were fit to a log normal distribution as described previously (Chirita et al., 2005).

All linear and non-linear regression fits are reported ± standard error of the estimate. The probability that an observed difference in the mean of replicates was statistically significant was determined by paired \( t \) test.

### 4.3 Results.

#### 4.3.1 CR induces tau fibrillization \textit{in vitro}.

Experimentation Aromatic heterocycles thiazin red, thioflavin S, and CR each are capable of inducing tau fibrillization \textit{in vitro} (Chirita et al., 2005). In preliminary experiments, however, only CR entered HEK-293 cells over a period of days on the basis of color uptake (data not shown). Therefore, CR was used as tau fibrillization inducer in all subsequent experiments. To establish the concentration optimum for tau fibrillization
in vitro, varying concentrations of CR were incubated (37°C for up to 96 h) with 1 µM His₆-htau40 and assayed for fibrillization using quantitative EM. No filaments formed in the absence of CR under these assembly conditions (Fig. 4.1A). In contrast, the presence of CR induced filaments that grew as twisted ribbons with maximum widths of 19 ± 4 nm ($n = 50$), minimum widths of 8 ± 2 nm ($n = 50$), and half periodicity of 114 ± 16 nm ($n = 32$) (Fig. 4.1B). CR concentration dependence was biphasic, and the data could be fit to a log-normal concentration effect profile to yield an estimate of optimum potency at 19.9 ± 0.5 µM CR (Fig. 4.1D). These data suggest that CR was ~6-fold more potent than thiazin red as a tau fibrillization inducer (Chirita et al., 2005), and established an optimum CR concentration range of 10 – 20 µM.

The time course of CR-mediated aggregation (1 µM His₆-htau40) was estimated at 10 µM CR. At these reactant concentrations, fibrillization proceeded without a perceptible lag and yielded well resolved filaments for the first 48 h. After that time, however, filaments formed tangled masses that were impossible to quantify by EM methods (Fig. 4.1C). When the first 48 h of reaction was modeled as a first order approach to plateau using the monomolecular equation (eq 1), $k_{\text{app}}$, the first order rate constant for growth of the filament population was $0.28 \pm 0.03$ h⁻¹. Together these data demonstrate that fibrillization of micromolar concentrations of full length tau protein can be rapidly induced by 10 µM CR under near physiological conditions.

To test the solubility of CR-induced tau filaments in vitro, aliquots from tau assembly mixtures used for the EM study were centrifuged at 100,000g for 1 h and separated into supernatant and pellet fractions. Tau protein was then detected by
immunoblot analysis using a monoclonal antibody, Tau5, that binds a continuous, non-phosphorylated epitope common to all human tau species (Carmel et al., 1996). The results showed that tau gradually shifted from the soluble to the pellet fraction over time in the presence of CR (Fig. 4.2A). The rate of loss of soluble tau was well fit by an exponential decay, consistent with the first order aggregation model (Fig. 4.2B). The apparent first order rate constant for loss of soluble tau, $k_{\text{app}}$, was $0.10 \pm 0.02$ h$^{-1}$. This value was not statistically different from the $k_{\text{app}}$ determined from appearance of tau in the pellet fraction ($0.12 \pm 0.07$ h$^{-1}$), but was significantly lower than $k_{\text{app}}$ determined from length measurements (Fig. 4.1). These results indicate that sedimentation is a less sensitive assay method than electron microscopy (e.g., it fails to capture short filaments), or that length and mass do not have a direct relationship (e.g., owing to heterogeneous nucleation occurring along the length of filaments). Overall, however, sedimentation was an adequate method for estimating tau aggregation kinetics in the presence of CR and one that was applicable to cellular models.

### 4.3.2 Congo red induces tau aggregation in cell culture.

To determine whether CR could induce tau aggregation in cell culture, HEK-293 cells stably overexpressing the longest human tau isoform (tau cells) were incubated with varying concentrations of CR for up to 7 days. HEK-293 cells were chosen for this experiment because they tolerate high-level tau overexpression ($5.3 \pm 0.6$ pg/cell, $n = 4$ determinations, data not shown) without evident toxicity (Ko et al., 2004). Cell lysates were then prepared in nonionic detergent NP-40, separated into soluble and insoluble
fractions by ultracentrifugation (1 h at 100,000g), and subjected to immunoblot analysis using anti-tau and anti-α-tubulin monoclonal antibodies. Insolubility in both ionic and nonionic detergents (i.e., at low temperatures) is a characteristic of authentic AD-derived tau filaments (D'Souza et al., 1999; Sergeant et al., 2003). Results showed that Tau5 immunoreactivity appearing in the pellet fraction had a biphasic dependence on CR concentration (Fig. 4.3A). Insoluble tau was not a result of cosedimentation with microtubules, because lysates were prepared under microtubule depolymerizing conditions where most tubulin remained in the soluble fraction (Fig. 4.3B). The optimum CR concentration for inducing insoluble cellular tau was similar to the optimum determined in vitro (Fig. 4.3C).

To characterize the time course of tau aggregation, tau cells were treated with 10 μM CR for up to 14 days, then subjected to centrifugal fractionation and immunoblot analysis as described above. Results with monoclonal antibody Tau5 showed that insoluble tau accumulated without a lag and approached plateau after 7 days of treatment (Fig. 4.4A). When modeled as a first order aggregation reaction, the rate constant for growth of the insoluble tau pool, $k_{app}$, was 0.006 ± 0.002 h$^{-1}$. The appearance of insoluble tau was accompanied by an overall increase in total tau levels, with accumulation of insoluble tau accounting for much of the increase (Fig. 4.4D). After 7 days treatment, >30% of total tau in the cell was insoluble (Fig. 4.4E), with the model predicting maximal aggregation asymptotically approaching 38 ± 6% of total tau. These results suggest that CR was capable of driving robust aggregation in this cellular model.
Tau dissociates from microtubules (Biernat et al., 1993; Bramblett et al., 1993) and can aggregate (Sato et al., 2002) in response to high level phosphorylation in cell culture. To determine whether hyperphosphorylation of tau accompanied CR-mediated tau aggregation, lysate fractions were probed with monoclonal antibody PHF1, which binds tau phosphorylated at S\(^{396}/S^{404}\) (Otvos et al., 1994). These sites were analyzed because their occupancy changes in response to tau protein kinases thought to contribute to tau hyperphosphorylation including GSK3 (Reynolds et al., 2000), Cdk5 (Lund et al., 2001), and CK1 (Ko et al., 2004). Results showed that basal levels of PHF1 immunoreactivity in the soluble fraction did not change significantly over 14 days of CR treatment (10 \(\mu\)M CR; Fig. 4.4B). In the insoluble fraction, PHF1 reactivity rose in tandem with Tau5 immunoreactivity (Fig. 4.4B). These results suggest that CR-induced aggregation is unrelated to occupancy of the PHF1 phosphorylation sites.

To characterize the morphology of aggregates, wild-type HEK-293 cells or tau cells were plated on coverslips and then treated with 10 \(\mu\)M CR up to 7 days. Tau aggregates were then visualized by confocal immunofluorescence microscopy using the Tau5 antibody. Before addition of CR, Tau5 staining localized to the cell periphery as found previously for N-terminal tau fragments expressed at much lower levels in other cell types (Brandt et al., 1995). After only 1 day with 10 \(\mu\)M CR, however, the distribution shifted and small tau-positive inclusions could be detected in the cytoplasm (Fig. 4.5). After 7 days in culture, cytoplasmic inclusions dominated the staining pattern (Fig. 4.5).
To characterize the ultrastructure of these inclusions, the sarksosyl insoluble fraction of lysates prepared from tau cells treated for 7 days with 10 µM CR were subjected to immunogold labeling with anti-tau antibody Tau12 and viewed in an electron microscope. Both isolated and tangled masses of Tau-12 positive filaments were observed (Fig. 4.6). The morphology of the latter was similar to in vitro aggregates incubated for 96 h (Fig. 4.1C). Together these results suggest that the fibrillization-inducing activity of CR is similar in vitro and in cells.

4.3.3 Congo red induced tau aggregation reduces the ability of tau to bind tubulin.

To determine whether CR-induced tau filament formation modulated tau function, the ability of His₆-htau40 treated with CR in vitro for up to 24 h to bind purified tubulin was examined. Complexes were isolated by immunoprecipitation with α-tubulin antibody rather than sedimentation to avoid potential cosedimentation of microtubules and tau filaments. In the absence of CR, tau co-immunoprecipitated with tubulin (Fig. 4.7A). The addition of CR led to time dependent loss in tubulin-bound tau levels with an initial rate that was faster than the rate of loss of tau from the soluble fraction. These data suggest that the product of CR/tau interaction has a low affinity for tubulin.

To confirm these findings, lysates prepared from tau cells grown in the presence or absence of CR for 7 days were subjected to tubulin immunoprecipitation with anti-α-tubulin antibody and immunoblot analysis with Tau5. Over this time period, the presence of CR yielded a doubling of total tau levels relative to cells incubated in its absence (Fig. 4.8). Nonetheless, the amount of endogenous tau that immunoprecipitated
with endogenous tubulin decreased over 30% (Fig. 4.8). Together these data suggest that the change in tau conformation or aggregation state induced by CR diminished tubulin binding affinity relative to tau monomer in random coil conformation.

**Tau aggregation and cell viability** – CR is well tolerated in some cell types, with concentrations as high as 100 µM being non-toxic over short time periods (Apostol et al., 2003; Bao et al., 2004). To characterize CR toxicity under the conditions of the current study, the effect of incubation of tau cells with up 0 – 100 µM CR for up to 14 days on cell numbers was measured. Results showed that CR toxicity was both time and concentration dependent. All concentrations were well tolerated for up to four days, but ≥7 day incubations resulted in statistically significant decreases in cell numbers at ≥30 µM CR. The concentration of 10 µM CR, which induces filament formation, did not significantly affect tau cell viability over 14 days in culture (Fig. 4.8). These data suggest that the amounts of tau aggregation induced by 10 µM CR are not toxic to tau cells over 14 days exposure.

Previous studies have shown that tau turnover is mediated by the ubiquitin-proteasome system (David et al., 2002), and that tau aggregates bind and inhibit this system (Keck et al., 2003). To determine whether the toxicity of tau aggregation could be unmasked by further compromising the ubiquitin-proteasome system, both wild-type HEK-293 and tau cells were treated with CR for 7 days, with the cells challenged further by the addition of ALLN or vehicle after the first 4 days in CR. ALLN is a cell-permeable inhibitor of the proteasome and also other proteases (Vinitsky et al., 1992). In
the presence of CR, cell counts for wild type HEK-293 and tau cells did not differ significantly ($p < 0.05$) from each other or from cells incubated with DMSO vehicle alone (Fig. 4.10). These data suggest that tau aggregation induced by CR in tau cells did not affect viability relative to wild-type cells devoid of any tau, and that CR itself was not toxic under these conditions (Fig. 4.10). ALLN treatment alone led to small decreases in the numbers of tau cells relative to HEK-293 cells that were not statistically significant ($p < 0.05$). In contrast, the presence of both CR and ALLN led to a statistically significant decrease in tau cell numbers relative to HEK-293 cells ($p < 0.01$). These data suggest that tau aggregation alone is not toxic to HEK-293 cells over short time spans in culture, but that tau aggregation in the presence of exogenous stress in the form of ALLN can lead to detectable losses in viability in within 7 days.

4.4 Discussion.

These data suggest that tau fibrillization agonists such as CR can be leveraged to create novel cellular models of tauopathy. The approach has several advantages. First, it facilitates study of full-length tau isoforms without resorting to aggregation-prone truncation (Khlistunova et al., 2006) or tauopathy mutants (DeTure et al., 2002; Vogelsberg-Ragaglia et al., 2000). Full-length tau is central to AD progression because truncation, while appearing early in disease, occurs well after filament deposition is underway (Guillozet-Bongaarts et al., 2005). Second, aggregation proceeds without the need for tau hyperphosphorylation, and so avoids the nonspecific changes in phosphorylation of cellular proteins that accompany addition of phosphatase inhibitors.
(e.g., okadaic acid, (Kuret et al., 1997)) or overexpression of specific protein kinases (Sato et al., 2002). Finally, agonists drive the highest levels of aggregation yet achieved in a cellular model, with >30% of cellular tau entering the detergent-insoluble particulate fraction within 7 days. The kinetic treatment used here predicts that the maximal amounts of detergent insoluble tau at reaction plateau would attain nearly 40% of total cellular tau. This far exceeds levels achieved using tauopathy mutants (DeTure et al., 2002; Vogelsberg-Ragaglia et al., 2000) and more closely models the high levels of aggregated tau seen in AD neurons (Khatoon et al., 1992). CR-mediated increases in total tau levels correlated with appearance of detergent insoluble tau, suggesting that the observation results from sequestration of tau rather than increases in tau expression. Tau expression does not change in AD (Hyman et al., 2005; Yasojima et al., 1999), suggesting that this mechanism may be relevant for disease as well. The shift in tau levels to the new steady state may be an example of the plateau principle (Goldstein et al., 1974), which describes the kinetics of accumulations that are cleared by first order processes (e.g., protein turnover; (Berlin and Schimke, 1965)). Assuming that the rate of tau biosynthesis is constant, and that the rate of CR uptake is fast relative to aggregation, then the rate of approach to the new steady state should approximate the turnover rate of the aggregated species. The plateau principle together with the rate data in Fig. 4.4 predict that tau aggregates have a half-life of ~115 h in HEK-293 cells, which is approximately 1 – 2 orders of magnitude longer than the half-life for non-aggregated tau (David et al., 2002; Vila-Ortiz et al., 2001).
The aggregation agonist model also has several limitations. First, CR-induced tau filaments differ from authentic tissue-derived PHF with respect to morphology. Despite similarities in overall dimensions, CR-induced filaments are more flexible and have a longer periodicity of twist than authentic PHF. Although filament morphology varies in different tauopathies (e.g., (King et al., 2001; Ksiezak-Reding et al., 1996)), the significance of morphology for disease progression is poorly understood. Second, the current model relies on tau concentrations of ~5.6 pg/cell. Assuming a typical HEK293 cell volume of ~4 pl (Chambard and Ashmore, 2003; DeTure et al., 2002), these levels correspond to intracellular tau concentrations (~25 µM) well above estimates of physiological tau concentrations in cells (Drubin et al., 1985) and also adult brain homogenates (Khatoon et al., 1992). In either case, however, the amount of free tau is unknown. The ability of agonist inducers to support tau aggregation as low as 200-300 nM htau40 (Chirita et al., 2005) suggests that it may be possible to induce tau aggregation at more physiological bulk tau concentrations in future. Third, CR-induced aggregation is not associated with phosphorylation of the PHF1 sites beyond basal levels. The absence of hyperphosphorylation may be useful for assessing aggregation in the absence of protein phosphorylation-mediated effects, but may not capture potential toxicity of aggregates. For example, high level accumulation of phospho-tau aggregates have been suggested to sequester PIN1 as part of their mechanism of toxicity (Lu et al., 1999). Still, there are many sites beyond Ser\textsuperscript{396}/Ser\textsuperscript{404} that are at least partially occupied in cells and these may change in the presence of CR. Fourth, CR appears to drive homogeneous nucleation, whereas the association of PHFs with cytomembranes (Gray et
al., 1987) is more consistent with a heterogeneous nucleation mechanism (Abraha et al., 2000). Experience with β-amyloid peptide suggests that it is the presence of active fibrillization, rather than filaments per se, that is toxic for cells (Wogulis et al., 2005). If so, then site of nucleation may be critical. Heterogenous interactions may contribute to toxicity in part by controlling the location of nucleation and extension reactions, and homogeneous nucleation paradigms may miss this aspect of toxicity. Finally, the current model cell line, HEK-293, may not recapitulate events in neurons and glia. However, it may be possible to port aggregation agonists to these cell types for modeling in future.

With these limitations in mind, the results found here in HEK-293 cells are significant in two respects. First, they indicate that the initial rate of loss of tubulin binding affinity exceeds the rate of disappearance of soluble tau in the presence of CR. This suggests that small detergent-soluble tau aggregates formed early in the reaction pathway, or perhaps the conformation of tau monomer stabilized by the presence of CR, has a lower affinity for tubulin binding than random coil tau monomer. The changes in tubulin affinity appeared unrelated to tau hyperphosphorylation, at least as judged by occupancy of the PHF1 sites. Although it has been proposed that tau fibrillization begins in association with microtubules (Ackmann et al., 2000), the observed incompatibility between tau aggregation and microtubule binding suggests that microtubule dissociation precedes aggregation. This would not be surprising, because the same segment of tau that mediates tubulin binding also mediates homo-polymerization (Buee et al., 2000).

Second, the results have implications for tau filament toxicity. In the HEK-293 cell background, tau aggregation appearing in the cell cytoplasm did not affect cell
growth over a 14-day period. In the presence of protease inhibitor ALLN, however, aggregate formation led to a statistically significant decrease in cell viability that was dependent on the presence of tau protein. Among the targets of ALLN is the proteasome, which plays a major role in the ATP-dependent turnover of many proteins including tau (David et al., 2002; Petrucelli et al., 2004). Proteasome function can be impaired by protein aggregates, suggesting a positive-feedback loop through which gradual aggregation could lead to both toxicity and also additional aggregate formation (Bence et al., 2001; Bennett et al., 2005). The ALLN-dependent loss of tau cell viability in the presence of CR is consistent with this model. The results also are consistent with the lack of acute toxicity associated with NFT formation (Andorfer et al., 2005; Santacruz et al., 2005). In AD, however, >90% of cortical tau aggregation localizes to neuropil threads and dystrophic neurites associated with neuritic plaques, indicating that most tau aggregation occurs in neuronal processes rather than in cell bodies (Mitchell et al., 2000). These aggregates may affect processes other than viability that are nonetheless important for disease progression, including retraction and loss of dendritic arbors (Falke et al., 2003) and synaptic contacts (Hamos et al., 1989).

It will be important to examine tau aggregate in a neuronal background so as to examine these aspects of toxicity. The method of inducing fibrillization described here should be portable to neuronal cultures. Because dyes such as CR support tau aggregation at ~200-300 nM levels (Chirita et al., 2005), it may be possible to induce aggregation of endogenous neuronal tau at physiological concentrations.
In summary, agonist mediated tau aggregation recapitulates salient features of tauopathy, including increases in total tau concentration, dissociation from microtubules, and formation of filaments in cytoplasm. A weak toxic phenotype can be observed in HEK293 cells that are exacerbated by protease inhibitor ALLN. The results suggest that tau aggregates forming within cell bodies may synergize with other stresses and contribute to disease progression without causing acute cell death.

4.5 Summary.

Intracellular aggregation of the microtubule-associated protein tau into filamentous inclusions is a defining characteristic of Alzheimer’s disease. Because appearance of tau-aggregate bearing lesions correlates with both cognitive decline and AD neurodegeneration, it has been hypothesized that tau aggregation may be directly toxic to cells that harbor them. Testing this hypothesis in cell culture has been complicated by the resistance of full-length tau isoforms to aggregation over experimentally tractable time periods. To overcome this limitation, a small-molecule agonist of the tau aggregation reaction that is active in vitro, Congo red, was applied HEK-293 cells stably expressing full-length tau isoform htau40. Formation of detergent insoluble aggregates was both time and agonist concentration dependent. At 10 µM Congo red, detergent insoluble aggregates appeared with pseudo-first order kinetics and a half-life of approximately 5 days. By seven days in culture, total tau levels increased two-fold, with approximately 30% of total tau converted into detergent-insoluble aggregates. Agonist addition also led to rapid losses in the tubulin binding activity of tau, although occupancy of
phosphorylation sites Ser$^{396}$/Ser$^{404}$ did not change during the course of experimentation. On the basis of viable cell counts, aggregation was not toxic up to 14 days in culture. However, the presence of proteasome inhibitor N-acetyl-leu-leu-norleu-al unmasked a tau-aggregate dependent loss of viability. The results, which establish a new approach for analysis of tau aggregation in cells independent of tau hyperphosphorylation, suggest that conformational changes associated with aggregation are incompatible with microtubule binding, and that aggregation can be toxic in the presence of cellular stress that compromises proteasome function.
Full-length human tau isoform His<sub>6</sub>-htau40 (1 μM) was incubated (37°C) under standard conditions with 0, 1, 3, 10, 30, or 100 μM CR for various times up to 96 h. Resultant tau filaments were visualized and average total length adsorbed per field (Γ<sub>f</sub>) quantified by transmission electron microscopy. Although filaments never formed in its absence A, (0 μM CR, 24 h), tau filaments with twisted ribbon morphology formed in B, the presence of CR (10 μM CR, 24 h). C, over multiple days (10 μM CR, 96 h), filaments clumped together to form tangled masses. D, at reaction plateau (24 h incubation), the CR concentration dependence of fibrillization was biphasic. Each data point represents the mean ± SD of triplicate determinations whereas the solid lines represent best fit to a log-normal distribution. E, at constant tau (1 μM) and CR (10 μM) concentrations, fibrillization increased hyperbolically with time without any detectable lag. Each data point represents the mean ± SD of triplicate determinations whereas the solid lines represent best fit to a monomolecular growth function (eq 1). Bars = 100 nm.
Figure 4.2. Centrifugation assay for CR-mediated tau fibrillization.

Tau assembly reactions prepared as described in Figure 1 were incubated up to 4 days, centrifuged (1 h at 100,000g) at room temperature, and the resultant pellet and supernatant fractions subjected to SDS-PAGE and immunoblot analysis using monoclonal antibody Tau5. A, B, examples of immunoblots conducted on supernatant and pellet fractions, respectively. Fibrillization results in a time dependent shift in tau from supernatant to pellet fractions. C, densitometric quantitation of data in panels A and B for supernatant (○) and pellet (●) fractions. Each data point represents the mean ± SD of quadruplicate determinations whereas the solid lines represent best fit to monomolecular growth (●; eq 1) or exponential decay (○; eq 2) functions.
Figure 4.3. CR induces tau aggregation in cell culture.

Tau cells treated with different concentrations of CR for 7 days were harvested, lysed, and separated into supernatant and pellet fractions by centrifugation (1 h x 100,000g) as described in Experimental Procedures. Representative immunoblots for A, total tau (Tau5 epitope) and B, α-tubulin (DM-1A epitope) levels are shown. Under these conditions, microtubules were unstable and all tubulin appeared in the supernatant fraction. In contrast, the amount of tau in the pellet fraction was dependent on CR concentration. C, total tau detected by Tau5 antibody in pellet fraction was quantified by densitometry and plotted versus CR concentration. Each bar represents the mean ± SD of four independent experiments. The CR concentration dependence for inducing insoluble tau protein in these cells was biphasic.
**Figure 4.4. CR induces insoluble tau aggregates in a time-dependent manner without hyperphosphorylation.**

Tau cells treated with 10 µM of CR for up to 14 days were harvested, lysed, and separated into supernatant and pellet fractions by centrifugation (1 h x 100,000g) as described in Experimental Procedures. Representative immunoblots for A, total tau (Tau5 epitope), B, phospho-tau (PHF1 epitope), and C, α-tubulin levels are shown. Under these conditions, microtubules were unstable and all tubulin appeared in the soluble fraction. In contrast, the amount of tau in the pellet fraction was dependent on time of treatment with CR but was largely unrelated to levels of PHF1 immunoreactivity. D, E, Tau5 immunoreactivity was quantified by densitometry for all cellular tau (pellet + supernatant; ●) and pellet tau (○), and pellet tau as percentage of all cellular tau (■). Each point represents the mean ± SD of four independent experiments. CR treatment lead to time-dependent increases in total cellular tau levels, largely owing to formation of insoluble tau. After 7 days incubation, ~30% of total cellular tau was isolated in the pellet fraction.
Figure 4.4. CR induces insoluble tau aggregates in a time-dependent manner without hyperphosphorylation.
Figure 4.5. CR-treated tau cells generate intracellular inclusions.

Wild type HEK-293 and tau stable cells were plated onto coverslips for 24 h, then treated with 10 µM CR for up to 7 days. Cells were then fixed with methanol, immunostained with monoclonal antibody Tau5, and visualized by confocal microscopy. Before (data not shown) and immediately after addition of CR (day 0), tau was detected at the cell periphery. Continued incubation resulted in a shift in distribution from the periphery to cytoplasmic inclusions.
Figure 4.6. Cellular CR-induced tau aggregates consist of filaments.

Tau aggregates were isolated from CR-treated tau cell as described in Experimental Procedures and visualized by immuno-electron microscopy using monoclonal antibody Tau12 and gold-labeled secondary antibody. Scale bar is for all three panels. Cellular CR-induced aggregates appeared as clumps of filaments.
Figure 4.7. CR induced tau aggregation decreases tubulin binding *in vitro*.

Aliquots of the time course reaction shown in Fig. 1 (i.e., the 0, 1, 4, 8, 24 h time points) were taken before centrifugation and incubated with 1 mg/ml purified bovine tubulin *in vitro* (30 min at 37°C). The relative amount of tau bound to tubulin was then estimated by immunoprecipitation with anti-α-tubulin antibody and immunoblot analysis with the Tau5 antibody as described in Experimental Procedures. A, A representative immunoblot probed with Tau5. Input tubulin was visualized after SDS-PAGE by staining with Coomassie blue. CR treatment induced a time dependent loss in the ability of His6-htau40 to bind exogenous tubulin. B, densitometric quantitation of panel A (and its replicates). Each point (○) represents the relative amount of tau coimmunoprecipitated with tubulin expressed as percent control (time 0), whereas the solid line is drawn to aid visualization. For comparison, the amount of soluble tau present at each time point (●; from Fig. 1) also is shown, with the solid line corresponding to the best fit to an exponential decay (Eq. 2). Tubulin binding activity declined rapidly relative to loss of soluble His6-htau40.
Figure 4.8. CR-induced tau aggregation decreases tubulin binding in tau cell culture.

Aliquots of the lysates prepared from CR-treated tau cells shown in Fig. 3 (i.e., the 0 and 7 day time points) were taken before centrifugation and the relative amount of tau bound to endogenous tubulin was estimated by immunoprecipitation with anti-α-tubulin antibody followed by immunoblot analysis as described in Experimental Procedures. A, a representative immunoblot probed with Tau5. CR treatment induced a time dependent loss in the ability of endogenous tau to bind endogenous tubulin. Input tubulin was visualized by immunoblot analysis with anti-α-tubulin antibody. B, densitometric quantitation of panel A (and its replicates), where each bar represents the amount of tau coimmunoprecipitated with tubulin expressed as percent of the value at time 0. The amount of total tau present at each time point (from Fig. 4) also is shown for comparison. Although CR treatment led to increased total cellular tau levels, the amount of tau capable of binding tubulin decreased.
Figure 4.9. CR toxicity in tau cells.

Tau cells were plated onto coverslips for 24 h, then treated with varying concentrations of CR or DMSO vehicle for up to 14 days. Cells were then washed three times with PBS, harvested by trypsinization, and counted. Cell numbers were normalized to control populations (i.e., cells treated with vehicle alone). Each bar represents the mean ± SD from four independent experiments. CR toxicity in tau cells is both time and concentration dependent. *, $p < 0.05$; #, $p < 0.01$ when compared with tau cells incubated with vehicle alone.
Figure 4.10. CR toxicity in tau cells.

Wild type HEK-293 and tau cells were plated onto coverslips for 24 h, then treated with CR (10 µM) or DMSO vehicle for 4 days. ALLN (26 µM) or EtOH vehicle alone were then added to the medium for another 3 days. Cells were then washed three times with PBS, harvested by trypsinization, and counted. Cell numbers were normalized to control populations (i.e., cells treated with vehicle alone). Each bar represents the mean ± SD from four independent experiments (hollow bar, HEK-293; black bar, tau cells). Although statistically significant ($p < 0.05$) although neither CR-treated nor ALLN-treated tau cells differed (at $p < 0.05$) in viability relative to CR-treated wild-type HEK-293 cells, the combination of CR and ALLN unmasked a statistically significant tau-dependent toxicity. *, $p < 0.05$ when compared with identically treated HEK-293 cells.
CHAPTER 5

5 IDENTIFICATION OF NOVEL SUBSTRATE AND INHIBITOR OF HUMAN CASEIN KINASE-1

5.1 Introduction.

Casein kinase 1 (CK1) is a family of structurally conserved protein kinases characterized by monomeric structure, unusually high isoelectric point, and constitutive phosphotransferase activity selective for Ser/Thr residues located in or adjacent to anionic segments of substrate proteins (reviewed in Refs. (Behrend et al., 2000; Gross and Anderson, 1998)). Each CK1 family member consists of a highly conserved N-terminal catalytic domain linked to C-terminal segments of differing length and amino acid sequence. In humans, CK1 is encoded by six distinct genes (Ckiα, γ1, γ2, γ3, δ, and ε), with further structural complexity attained by alternative splicing.

CK1 enzymes are found in all eukaryotes. CK1 family members phosphorylate cytoskeletal proteins such as spectrin, troponin, myosin, ankyrin, and α-synuclein (Behrend et al., 2000; Okochi et al., 2000; Simkowski and Tao, 1980; Singh et al., 1995; Singh et al., 1996), but also non-cytoskeletal proteins such as SV40 T antigen (Cegielska et al., 1998), p53 (Behrend et al., 2000; Knippschild et al., 1997) and β-catenin (Marin et
Phosphorylation of these substrates is thought to modulate diverse physiological functions such as vesicular trafficking, DNA repair, cell cycle progression, and cytokinesis (Gross et al., 1997).

Access to substrates is controlled in part by the subcellular distribution of individual CK1 isoforms. For example, in budding yeast, CK1 homologs Yck1p and Yck2p selectively associate with cell surface membranes (Vancura et al., 1994) where they gain access to membrane-bound substrates including mating type receptors Ste2p and Ste3p (Feng and Davis, 2000; Hicke et al., 1998) and components of the permeases and sensors involved in the detection and transport of extracellular nutrients (Abdel-Sater et al., 2004; Marchal et al., 2000; Spielewoy et al., 2004). In each case, phosphorylation precedes ubiquitination and subsequent trafficking or proteasome-mediated turnover of substrate.

Interaction of CK1 with protein turnover machinery may also contribute to neurodegenerative diseases that accumulate misfolded protein within affected neurons. In AD tissue, Ckiδ levels are elevated (Ghoshal et al., 1999; Yasojima et al., 2000), and CK1 isoforms colocalize with ubiquitinated cytoplasmic lesions including both granulovacuolar degeneration bodies and the tau filaments of neurofibrillary tangles (Ghoshal et al., 1999). The appearance of CK1 in these lesions correlates strongly with cognitive decline in longitudinal studies of AD progression (Abraha et al., 2000), suggesting that CK1 may contribute to hyperphosphorylation and subsequent aggregation of resident proteins. Consistent with this observation, tau filaments isolated from AD brain contain up to 0.5% CK1 on a weight basis (Green and Bennett, 1998). These
observations led to the hypothesis that CK1 enzymes regulate tau phosphorylation in vivo, and that their upregulation in level or activity contributes to tau hyperphosphorylation in disease (Ghoshal et al., 1999).

Here we test this hypothesis by examining the ability of one CK1 isoform, Ckiδ, to phosphorylate tau under basal and overexpression conditions in situ. Also, a human brain cDNA library was subjected to yeast two-hybrid screening using the human Ckiδ isoform as bait to identify proteins capable of regulating CK1. All positive clones encoded a previously uncharacterized protein which we term as CK1-binding protein (CK1BP). We also described the identification and characterization of CK1BP, a homolog of dysbindin (a protein involved in protein trafficking and lysosome biosynthesis and implicated in muscular dystrophy and schizophrenia).

5.2 Materials and methods.

Materials. Enhanced chemiluminescence (ECL) detection reagents and [γ-32P] ATP were from GE Healthcare (Waukesha, WI), Ni2+-NTA agarose beads from Qiagen (Valencia, CA), protein G Plus-Agarose from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). HEK-293 cells were purchased from ATCC (Manassas, VA). TransFast™ Transfection Reagent was from Promega (Madison, WI). Partially hydrolyzed casein (5% solution) and protease inhibitors containing (final concentration) 1 mM 4-(2-aminoethyl)-bezenesulfonylfluoride, 0.8 µM aprotinin, 40 µM bestatin, 20 µM leupeptin, 15 µM pepstatin A, 14 µM L-transepoxy succinyl-leucylamido-[4-guanidine] butane, and 26 µM
N-acetyl-leu-leu-norleu-al) were obtained from Sigma (St. Louis, MO). Synthetic peptide substrate RRDDEEDEEMSETADGER (corresponding to residues 166-180 of rabbit skeletal muscle phosphatase inhibitor-2 with terminal Arg residues added to the natural peptide; (Marin et al., 1994)) was a gift from ICOS corporation (Bothell, WA). Full-length cDNAs encoding human Ckiα (CSNK1A lacking L and S inserts described in Ref. (Yong et al., 2000), Ckiδ (CSNK1D variant 1; Ref. (Kusuda et al., 1996)), Ckiε (CSNK1E; Ref. (Fish et al., 1995)), Ckiγ2 (CSNK1G2; Ref. (Kitabayashi et al., 1997)), Ckiγ3 (CSNK1G3; Ref. (Kusuda et al., 1998)), and dysbindin (DTNBP1 variant c; GenBank accession number NM183041) were obtained by PCR amplification of an adult human brain cDNA library prepared in pMyr vector (Stratagene, La Jolla, CA). cDNA encoding C-terminal truncated Ckiδ (Ckiδ-Δ317) was obtained through the same method with a TTA stop codon added downstream of the codon for His317.

**Antibodies.** Recombinant Polyclonal antibody against the C-terminus of p35 (C-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas monoclonal anti-α-tubulin antibody (Blose et al., 1984) was obtained from Sigma/Aldrich Chemicals (St. Louis, MO). Monoclonal antibodies AT8, Tau5, and PHF1 were obtained from Endogen (Woburn, MA), Dr. L.I. Binder, Northwestern University Medical School, and Dr. Peter Davies, Einstein College of Medicine, respectively. Monoclonal antibodies 128A (anti-Ckiδ) and 94.1 (anti-Ckiα) were the gifts of ICOS Corporation (Bothell, WA), whereas anti-Ckiε and 12CA5 were from BD Biosciences (San Jose, CA) and Roche Molecular Biochemicals (Indianapolis, IN), respectively. Peroxidase-labeled affinity purified anti-mouse secondary antibody was from KPL (Gaithersburg, MD).
**Yeast two-hybrid.** Screening was performed with an hSos/Ras recruitment system in Cdc25Hα cells (MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal+) (Aronheim et al., 1997; Broder et al., 1998) according to manufacturer’s instructions (Stratagene). The coding region of full-length Ckiδ was amplified by PCR, cloned into the BamHI site of the bait plasmid pSos, and co-transformed with an adult human brain cDNA library into *S. cerevisiae* cdc25Hα cells. Yeast clones that grew on glucose minimal media at 25°C and galactose minimal media at 37°C, but not on glucose minimal media at 37°C, were judged positive for interaction (Aronheim et al., 1997; Isakoff et al., 1998). Positive prey plasmids were isolated from yeast and sequenced in both directions using vector-specific primers at the 5’ and 3’ ends.

For directed two hybrid analysis, CK1BP, dysbindin, or dysbindin Δ1-83 cDNA was isolated and cloned into the pMyr vector at its EcoRI/XhoI sites. Human Ckiα, γ2, γ3, Ckiδ-Δ317 (Graves and Roach, 1995) and ε were amplified by PCR and cloned into pSos. Interaction between each of these CK1 constructs and pMyr-CK1BP was then tested in yeast cdc25Hα cells.

**Recombinant proteins.** All His6-tagged proteins (CK1BP, htau40, α-synuclein, dysbindin, and Ckiδ-Δ317) were expressed in *E. coli* using vector pT7C and purified as described previously (Carmel et al., 1996; Ko et al., 2004; Necula et al., 2003). CK1BP without the His6-tag was expressed in *E. coli* using vector pT7II (Carmel et al., 1994). Recombinant, non-tagged protein was purified after extract preparation (Carmel et al., 1994) by liquid chromatography. The extract was loaded directly onto a Sepharose-Q fast flow column equilibrated in buffer A (10 mM MOPS, pH 7.0, 0.1 mM EGTA).
containing 50 mM NaCl, washed with 5 bed volumes of Buffer A containing 150 mM NaCl, and eluted with Buffer A containing 300 mM NaCl. Fractions containing CK1BP were pooled and brought to 25% saturation with solid (NH₄)₂SO₄, loaded onto a phenyl-Sepharose column equilibrated in buffer B (50 mM sodium phosphate, pH 7.0, 0.1 mM EGTA) containing 1 M (NH₄)₂SO₄, washed with 5 bed volumes of Buffer B containing 800 mM (NH₄)₂SO₄, and eluted with Buffer B containing 400 mM (NH₄)₂SO₄. Fractions containing CK1BP were pooled, diluted with buffer C (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.1 mM EGTA) to lower the salt concentration, concentrated to 4 mL by centrifugal filtration (Centricon-30; Amicon, MA), and finally loaded directly onto a 180-mL column (1.6 x 91 cm) of Sephacryl S-200 HR gel filtration column equilibrated and run (30 mL/h) in Buffer C. Fractions containing CK1BP were pooled, concentrated, and stored at -20°C until used.

**Cell culture and transfection.** HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine serum, 100 U/ml penicillin G, 250 ng/ml amphotericin B, and 100 µg/ml streptomycin (37°C with 5% CO₂). A triple-HA tagged CK1BP construct was created by taking the triple HA-tag from pSLF173 (Forsburg and Sherman, 1997) as a XhoI/NotI fragment and ligating it into the pcDNA3.1 (Invitrogen) vector. CK1BP was amplified by PCR and cloned directly into PCR-BluntII-TOPO vector (Invitrogen). The construct was then digested with NotI/EcoRI and ligated into the pcDNA3.1 in frame with the HA tag. This plasmid was transiently transfected to HEK-293 cells using Lipofectamine 2000 (Invitrogen) according to the
manufacturer’s instructions, while pcDNA3.1/htau40, pcDNA3.1/Ckiδ, and pcDNA3.1/p25 were transiently transfected to HEK-293 cells using TransFast™ reagent according to the manufacturer’s instructions.

**His$_6$-pull down assay.** HEK-293 cells were harvested and disrupted in Lysis Buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 20 mM imidazole, 1 mM PMSF, 1% (v/v) Nonidet-40, 1 mM EDTA) containing 1x complete protease inhibitors (Ko et al., 2004). Cell debris was removed by centrifugation (10 min x 10,000g) at 4°C. Ni$^{2+}$-NTA-agarose beads were resuspended in lysis buffer in a ratio of 1:1 (v/v) and 40 µl of this slurry was incubated with 20 µg His$_6$-CK1BP or His$_6$-α-synuclein at 4°C for 1 h. The beads were pelleted by centrifugation and washed three times with lysis buffer. HEK-293 extracts (500 µg) were then added to the beads and incubated (2 h at 4°C). The beads were sedimented a second time, washed five times with lysis buffer, and finally resuspended in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and either stained with Coomassie brilliant blue R-250 or subjected to immunoblotting using monoclonal antibodies 128A, 94.1, or anti-Ckiε as described previously (Ko et al., 2004).

**Immunoprecipitation.** HEK-293 cells were harvested in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% NP-40, 5 mM EDTA, 0.5% sodium deoxycholate) containing 1x complete protease inhibitors, disrupted by sonication. After removal of cell debris by centrifugation (10,000g for 15 min), extracts (500 µg) were incubated (2 h at 4°C) with 2 µg of monoclonal antibody 128A (anti-Ckiδ) or 12CA5 (anti-HA tag). Protein G-agarose beads [60 µL of 25% (w/v) slurry] were then added to the samples and
incubation continued for 1 h at 4°C. The resultant immunocomplexes were collected by centrifugation, washed three times with RIPA Buffer, and subjected to immunoblot or \textit{in vitro} protein kinase assays as described below.

\textbf{In vitro kinase reactions.} Protein kinase activity of purified Ckiδ-Δ317 or of immunocomplexes derived from immunoprecipitation experiments was estimated by incubating each with various protein substrates in phosphorylation buffer (10 mM MOPS, pH 7.0, 50 mM NaCl, 4 mM MgCl\textsubscript{2}). When present, reactions contained varying amounts of CK1BP (0 – 2 µM). After preincubation for 15 min, reactions were initiated by the addition of 100 µM \[^{\gamma-32P}\]ATP (500 cpm/pmol), incubated for 30 min at 37°C, and terminated with SDS sample buffer. Following SDS-PAGE, the stained and dried gels were subjected to autoradiography on film or with a Molecular Imager FX Pro Plus multiimager system (Bio Rad).

\textbf{Circular dichroism.} Detergents Samples were prepared for CD analysis by desalting into 20 mM NaClO\textsubscript{4}, 100 mM H\textsubscript{3}BO\textsubscript{3}, pH 7.4. These buffer components were employed because of their transparency at far UV wavelengths (Schmid, 1997). Spectra were collected (178 – 260 nm) at 25°C using an AVIV model 202 CD spectrometer and a quartz cuvette with 0.01 cm path length. Four repetitive scans (4 s integration time, 1 nm step size, and 1 nm bandwidth) were recorded, averaged, and corrected for buffer-only blank without additional filtering or smoothing. Raw CD signals (in millidegrees) were converted to mean residue molar ellipticity \([\theta]_{MRW}\) in degree·cm\textsuperscript{2}·dmol\textsuperscript{-1} using the formula

\[ [\theta]_{MRW} = [\theta]_{obs}/(10 \times l \times c \times n), \]

where \([\theta]_{obs}\) is the observed ellipticity in millidegree, \(l\) is the
pathlength in centimeters, \( c \) is the molar concentration of protein, and \( n \) is the number of residues in the protein (35). Secondary structure composition of \( \alpha \) helix (normal and distorted), \( \beta \)-strand (normal and distorted), turns, and random coil were estimated from net spectra using CONTIN/LL (Provencher and Glockner, 1981), CDSSTR (Krishnamurthy and Johnson, 2004), and SELCON3 (Sreerama et al., 1999) algorithms. Resultant estimates were then averaged and presented ± S.D. (Sreerama and Woody, 2000).

**Hydrodynamic Analysis.** Analytical size-exclusion chromatography was performed (4°C) on a 15-mL (7.8 x 300 mm) TSK 3000 SWXL column equilibrated with buffer 10 mM HEPES, pH 7.0, 100 mM NaCl and operated at 0.5 mL/min. The hydrodynamic radius of CK1BP was estimated from calibration standards including bovine serum albumin (3.62 nm), ovalbumin (2.83 nm), bovine erythrocyte carbonic anhydrase (2.01 nm), and lysozyme (1.90) as described previously (Vancura et al., 1993).

Velocity sedimentation measurements were performed in linear 4 - 20% sucrose gradients containing 10 mM HEPES, pH 7.4, 150 mM NaCl. Standards included ovalbumin (4.3 s), bovine erythrocyte carbon anhydrase (3.2 s), myoglobin (1.9 s), and cytochrome C (1.6 s). After centrifugation (200,000g for 20 h at 4°C), gradients were fractionated and sedimentation coefficients were estimated as described previously (Vancura et al., 1993). Native molecular weight and frictional ratio were calculated from hydrodynamic parameters as described previously (Siegel and Monty, 1966) using partial specific volumes calculated from amino acid sequence (Perkins, 1986). Theoretical
hydrodynamic radii for CK1BP in native, molten globule, pre-molten globule, random coil, and denatured states were calculated from molecular weight using the empirical equations deduced as described previously (Chirita et al., 2005).

**Analytical methods.** Hyperbolic inhibition curves were fit to the rectangular hyperbola:

\[ y = \frac{ax}{b + x} \]  

(1)

where \( y \) is kinase activity determined at inhibitor concentration \( x \), and constant \( b \) corresponds to \( x \) at 50\% \( y_{\text{max}} \) (i.e., IC\(_{50}\)).

Probability values were determined by student’s \( t \) test for single comparison and one-way ANOVA with Tukey’s post hoc test for multiple comparisons. All analyses were performed using the InStat Software program.

Protein concentrations were determined for extracts using the Coomassie-blue binding method with bovine serum albumin as standard (Bradford, 1976), and for purified proteins by absorbance using extinction coefficients calculated from amino acid composition (Perkins, 1986).

Immunoblots were performed as described previously using peroxidase-labeled goat anti-rabbit or anti-mouse IgG as secondary antibody (Ko et al., 2004), and developed using the enhanced chemiluminescence.

### 5.3 Results.

#### 5.3.1 Ckiδ phosphorylates tau \textit{in vitro}.

To determine whether Ckiδ could directly phosphorylate tau, a truncated form of the human enzyme (Ckiδ-Δ317) was expressed and purified as a His\(_6\)-fusion protein and
used to phosphorylate tau protein \textit{in vitro}. Truncated enzyme was used because it was easily prepared after heterologous expression in \textit{E. coli} while retaining the protein kinase activity of full-length protein (Graves and Roach, 1995). Incubation of htau40 with purified Ckiδ-Δ317 in the presence of radioactive nucleotide substrate led to detectable phosphate incorporation within 30 min (Fig. 5.1A). Detection of total tau protein on Western blots using monoclonal antibody Tau5, the reactivity of which is not phosphorylation dependent (Carmel et al., 1996), revealed a substantial electrophoretic mobility shift by 4 h incubation (Fig. 5.1B). These slowly migrating tau species were strongly labeled by monoclonal antibodies AT8 and PHF1, which selectively bind phospho-tau residues S\textsuperscript{202}/T\textsuperscript{205} (Bramblett et al., 1993) and S\textsuperscript{396}/S\textsuperscript{404} (Otvos et al., 1994), respectively. These data show that CK1 isoform Ckiδ could directly phosphorylate tau protein, and that monoclonal antibodies AT8 and PHF1 could be used as probes for Ckiδ-mediated phosphorylation reactions.

5.3.2 Ckiδ phosphorylates tau \textit{in situ}.

To determine whether Ckiδ could modulate tau phosphorylation \textit{in situ}, HEK-293 cells were transiently transfected with plasmids encoding htau40 and either Ckiδ, p25 (an activator of Cdk5), or empty pcDNA vector. Transfection times of 24 h were employed because cells remained viable during this time frame in all studies reported herein (data not shown). Cdk5 activator p25 was included because of its ability to activate endogenous Cdk5 \textit{in situ} (Patrick et al., 1999) and therefore serve as an alternate source
of tau hyperphosphorylation activity. Total tau protein was detected with monoclonal antibody Tau5 whereas phospho-tau was detected with antibodies AT8 and PHF1. All cells transfected with htau40 constructs contained detectable levels of tau protein (Fig. 5.2A). When these cells were co-transfected with empty vector (i.e., in the absence of exogenous protein kinase), a portion of total tau was PHF1 and AT8 positive, reflecting basal levels of tau phosphorylation. Basal levels of Ckiδ also were substantial in these cells as demonstrated by Western analysis using monoclonal antibody 128A. However, cotransfection with DNA constructs encoding Ckiδ significantly increased the amounts of both Ckiδ and phospho-tau detectable by both the AT8 and PHF1 antibodies (Fig. 5.2A). These data recapitulate the pattern seen in vitro (Fig. 5.2A) and suggest that Ckiδ overexpression significantly increased tau phosphorylation in situ (Fig. 5.2B). Expression of p25 also induced a large increase tau phosphorylation, primarily owing to the absence of endogenous p25 in non-transfected cells (Fig. 5.2B).

To confirm that the increased levels of Ckiδ observed upon overexpression were accompanied by increases in phosphotransferase activity, Ckiδ was immunoprecipitated and subjected to in vitro kinase assays using htau40 and [γ-32P] ATP as substrates 24 h after transient transfection with Ckiδ/tau or pcDNA3.1/tau constructs. Radioactive assay reaction products were separated by SDS-PAGE, stained with Coomassie blue, and visualized on a phosphoimager. Although CK1 activity was found in both the Ckiδ/tau overexpressing and pcDNA3.1/tau control cells (Fig. 5.3A), quantitation of the data showed that Ckiδ overexpression led to ~2-fold increases in recoverable
phosphotransferase activity (Fig. 5.3B). These data confirmed that Ckiδ overexpression lead to increased Ckiδ activity within HEK-293 cells, but also showed that significant levels of Ckiδ activity were present under basal conditions.

5.3.3 Identification of CK1BP as a Ckiδ binding protein.

To identify binding partners of human Ckiδ, a human brain cDNA library was screened in a Sos recruitment yeast two-hybrid system. This screening paradigm was employed because of its ability to capture interactions occurring at membranes, the natural intracellular location of several CK1 isoforms (Behrend et al., 2000). Eighteen clones confirmed positive for interaction were obtained from $2.5 \times 10^6$ independent transformants. DNA sequence analysis revealed that all 18 positive clones were derived from a single open reading frame located on chromosome 20 at position 20q13.12 (C20orf35). Two variant transcripts have been identified for this locus. One transcript (GenBank accession number NM018478), identified in cDNA libraries prepared from human hypothalamus (Hall et al., 2000), encodes a protein predicted to contain 261 amino acid residues (27,830 Da; pI = 4.5). The second transcript (GenBank accession number BC012818) was also identified in hypothalamus as well as in signet-ring cell carcinoma (NEDO human cDNA sequencing project), in skeletal muscle (Telethon project B41), in placenta and in colon adenocarcinoma (Strausberg et al., 2002). Isoform 2 does not encode the first N-terminal 100 amino acids of isoform 1, yielding only the C-terminal 161 residues of this protein (17,493 Da; pI = 4.13). To clarify the relationship
between these known transcripts and the isolated Ckiδ interacting clones, sequence alignments were performed. Results revealed that the 18 interacting clones consisted of 11 unique sequences related to each other by small differences in their 5’ ends (Figure 5.4A), and that all were consistent with the shorter transcript being the source of Ckiδ-binding activity. The encoded 161-residue protein was therefore termed CK1BP (Casein Kinase 1 Binding Protein).

5.3.4 CK1BP structure.

The primary structure of human CK1BP is shown in Figure 5.4B. It consists of a strongly acidic sequence that is 39% identical and 63% similar to the C-terminal domain of dysbindin, a ubiquitous protein that has been implicated in the pathogenesis of Hermansky-Pudlak syndrome type 7 (Li et al., 2003) and familial schizophrenia (Straub et al., 2002). Three splice variants of human dysbindin have been identified from cDNA clones, each containing a coiled-coil domain that may mediate association with itself or other coiled-coil containing proteins (Benson et al., 2001; Benson et al., 2004). Variants differ by the presence or absence of a basic N-terminal domain and by alternative splicing of sequences in the acidic domain (Figure 5.4C). Similarly, CK1BP and the sequence derived from the longer transcript (GenBank accession number NM018478) differ by the presence or absence of a weakly basic N-terminal extension. Thus, on the basis of primary structure, CK1BP appears to be a member of the dysbindin protein family.

To assess CK1BP secondary structure content, recombinant CK1BP and His_{6}-tagged CK1BP were prepared from bacterial expression systems as described in
Experimental Procedures and subjected to circular dichroism spectroscopy. Both preparations yielded spectra typical of proteins with substantial random coil character (i.e., broad minima of ellipticity centered at 200 nm; Figure 5.5A). To quantify relative amounts of secondary structure, CD spectra were deconvoluted by three independent algorithms as described in Experimental Procedures (Figure 5.5B). On the basis of secondary structure content calculated from averages of the three fits, the two preparations contained ≤39% -helix and -strand conformation and did not differ significantly in secondary structure content from each other (p < 0.05). These data suggest that CK1BP contained limited secondary structure in solution, and that the presence of a polyhistidine tag did not significantly modify its folding.

To assess higher order structure, recombinant CK1BP was subjected to hydrodynamic analysis using a combination of gel filtration chromatography and velocity sedimentation. Results showed that under the near-physiological buffer conditions employed in the analysis, CK1BP had monomeric quaternary structure in solution with a hydrodynamic radius of $3.0 \pm 0.1$ nm and an axial ratio of ~8.7:1 (Table 5.1). Empirical formulae relating hydrodynamic radii to chain lengths have been derived for different folding states (Tcherkasskaya et al., 2003). Conversion of these theoretical hydrodynamic radii into frictional ratios $(f/f_o)_{obs}$ (Chirita et al., 2005) facilitates comparison among folding states on the basis of shape. Comparison of the experimentally determined $(f/f_o)_{obs}$ value for recombinant CK1BP with these theoretical predictions indicated that the recombinant preparation resides in a premolten globule-like
folding state. This state is characterized by loosely packed tertiary structure (Chaffotte et al., 1997). The molar ellipticities for CK1BP at 200 and 222 nm (-12522 and -2611 respectively) also were consistent with this assignment (Uversky, 1993). Together these data indicate that CK1BP is a monomeric structural homolog of dysbindin that adopts only partially folded secondary structure and limited tertiary structure when free in solution.

5.3.5 CK1BP is CK1 isoform selective.

In Human CK1 is composed of six distinct gene products, with further diversity created by alternative splicing. To determine whether CK1BP selectively bound individual CK1 isoforms, directed two-hybrid assays were performed with human pSos-Ckiα, δ, ε, γ2, γ3. The shortest Ckiα splice variant was used for this experiment because it lacked the L insert that mediates nuclear localization (Burzio et al., 2002; Fu et al., 2001), whereas Ckiγ1 was not analyzed owing to its close sequence similarity to Ckiγ2 and Ckiγ3 (Kusuda et al., 2000). Results confirmed that neither pMyr-CK1BP co-transformed with pSos empty vector nor pSos-Ckiδ co-transformed with pMyr empty vector rescued the growth defect of Cdc25Hα cells (Figure 5.6). In contrast, growth was restored when pMyr-CK1BP was co-transformed with pSos-Ckiγ2, γ3, and ε, but not Ckiα (Figure 5.6). These data suggest that interactions between CK1BP and CK1 family members are isoform selective.

CK1 family members are composed of homologous N-terminal catalytic domains fused to isoform-specific C-terminal extensions. To determine which domain mediated
interactions between CK1BP and Ckiδ, C-terminal truncation mutant Ckiδ-Δ317 was prepared and included in the directed 2-hybrid screen. This mutant was chosen for analysis because its protein kinase domain is intact (spanning residues 9-290) and folds properly in heterologous expression systems (Graves and Roach, 1995) whereas the bulk of the C-terminal extension (residues 318 – 415) is absent. Results showed that CK1BP interacted with Ckiδ-Δ317 similarly as with full-length Ckiδ (Figure 5.6), suggesting that its principal binding target is composed of the protein kinase catalytic domain or the short segment C-terminal to it.

5.3.6 CK1BP forms a complex with CK1 in vitro.

To confirm findings from two-hybrid screens, the ability of purified recombinant CK1BP to bind selected CK1 isoforms in cell lysates was determined in vitro. His$_6$-tagged CK1BP protein was used for these studies because it retained the folding properties of non-tagged recombinant CK1BP (Figure 5.5) while avidly binding Ni$^{2+}$-NTA beads. Analysis was limited to human Ckiα, δ, and ε because of the availability of specific monoclonal antibody probes for these three isoforms. Results showed that uncharged Ni$^{2+}$-NTA beads alone could not pull down any of these three CK1 isoforms (Figure 5.7). Similarly, beads charged with His$_6$-tagged α-synuclein (a protein unrelated in structure to CK1BP) did not selectively pull down CK1 isoforms. In contrast, beads charged with His$_6$-tagged CK1BP effectively pulled down Ckiδ and Ckiε but not Ckiα (Figure 5.7). These data recapitulate the interactions observed in yeast two hybrid experiments and demonstrate that CK1BP is a CK1 binding protein in vitro. Moreover,
they confirm that CK1BP does not directly bind Ckiα, suggesting that the failure of Ckiα to suppress the growth defect of Cdc25Hα cells in the presence of pMyr-CK1BP did not arise solely from deficiencies in the expression or folding of Sos-Ckiα fusion protein.

5.3.7 Full-length dysbindin does not interact with CK1 isoforms.

To determine whether dysbindin, a CK1BP structural homolog, retained CK1 binding activity, directed 2-hybrid screens were performed with pSos-Ckiα, δ, ε, γ2, γ3, or empty pSos vector as bait and pMyr-dysbindin (human variant c) or empty pMyr vector as prey. Surprisingly, full-length dysbindin was incapable of rescuing the growth defect of Cdc25Hα cells in the presence of any CK1 isoform (Figure 5.8). The result was not an artifact of yeast expression, because recombinant His6-tagged dysbindin was incapable of interacting with CK1 isoforms in pull down assays performed as described above for CK1BP (data not shown). These results suggested that either the dysbindin acidic domain was not functionally homologous to CK1BP, or that the coiled-coil domain common to all dysbindin variants but absent in CK1BP interfered with the ability of the acidic domain to interact with CK1.

To distinguish between these possibilities, the isolated acidic domain of dysbindin (i.e., the C-terminal sequence beginning with Met184 of dysbindin variant a shown in Figure 5.4B) was tested as prey in directed 2-hybrid screens with human Ckiα, δ, ε, γ2, γ3 as bait. Results show that the acidic domain of dysbindin could in fact rescue the growth defect of Cdc25Hα cells in the presence of Ckiγ2, γ3, δ, and ε, but not Ckiα.
Together these results suggest that the acidic domain of dysbindin could selectively interact with CK1 isoforms, and was a functional homolog of the acidic domain found in CK1BP.

5.3.8 CK1BP inhibits CK1 phosphotransferase activity.

To determine whether CK1BP binding affected the protein kinase activity of CK1, HEK-293 cell lysates were subjected to \textit{in vitro} autophosphorylation assays in the presence and absence of recombinant CK1BP. HEK-293 cells were chosen as the source of CK1 activity because they contain high basal levels of full-length human Cki\(\delta\) (Ko et al., 2004). Incubation of lysates with nucleotide substrate \textit{in vitro} gave rise to a pronounced Cki\(\delta\) band shift on immunoblots (Figure 5.9), consistent with the established role of autophosphorylation in modulating Cki\(\delta\) migration in SDS-PAGE (Graves and Roach, 1995). The presence of CK1BP antagonized this nucleotide dependent band shift (Figure 5.9).

These data suggest that CK1BP was capable of inhibiting Cki\(\delta\) autophosphorylation. To determine whether this reflected direct inhibition of Cki\(\delta\) catalytic activity, purified recombinant Cki\(\delta\)-\(\Delta317\) was incubated with various exogenous protein substrates in the presence of varying concentrations of recombinant CK1BP. Cki\(\delta\)-\(\Delta317\) was employed for this experiment because it is constitutively active, retains CK1BP binding activity, and is readily prepared from \textit{E. coli} expression systems for solution based assays (Graves and Roach, 1995). In addition, C-terminal truncation of Cki\(\delta\) eliminates most of its autoregulatory region, thereby reducing the modulatory effect of autophosphorylation on catalytic activity (Graves and Roach, 1995). Htau40 (\textit{i.e.}, full-
length four-repeat human tau protein; Ref. (Bramlett et al., 1993)) and human α-synuclein were used as protein substrate, because the former is a substrate for Ckιδ in situ (Ko et al., 2004), whereas the latter is phosphorylated by CK1 at a residue (Ser^{129}) thought to be important for protein aggregation in neurodegenerative disease (Lee et al., 2005; Okochi et al., 2000). Both substrates were phosphorylated by Ckιδ-Δ317 in vitro (Figure 5.10A, B). However, the phosphorylation of both substrates was inhibited by the presence of CK1BP, which was itself a substrate for Ckιδ-Δ317 (Figure 5.10A). Inhibition of phosphorylation was dose dependent, with IC_{50}s of 90 ± 8 nM and 57 ± 3 nM for tau and α-synuclein, respectively (Figure 5.10C). In the case of tau substrate, CK1BP-mediated inhibition was heat stable, suggesting that the small amount of higher order structure present in native CK1BP was not crucial for inhibitory activity (Figure 5.11). CK1BP at 1 μM was also capable of completely inhibiting phosphorylation of ~400 μM synthetic peptide substrate derived from phosphatase inhibitor-2 (residues 166-180; (Marin et al., 1994)) (data not shown). However, inhibition of casein phosphorylation was far less potent, requiring ~2.5 μM CK1BP to inhibit phosphorylation of 4 μM casein by 50% (data not shown). These data suggest that the association between CK1BP and Ckιδ-Δ317 is high affinity in the presence of some substrates, but that the ability of this association to inhibit protein kinase activity in vitro may depend on the nature and concentration of protein substrate.
5.3.9 CK1BP modulates CK1 phosphotransferase activity in cell culture.

To determine whether CK1BP could inhibit full-length Ckiδ in the presence of endogenous substrates within mammalian cells, Ckiδ from HEK-293 cells transiently transfected with either pcDNA-HA-CK1BP or pcDNA empty vector was immunoprecipitated with monoclonal antibody 128A and subjected to in vitro kinase assays. Immunoblots performed using an anti-HA monoclonal antibody confirmed that HA-tagged CK1BP was present in lysates prepared from cells transfected with pcDNA-CK1BP but not empty vector (Figure 5.12A). On the basis of immunoblot analysis with anti-Ckiδ monoclonal antibody 128A, the expression of endogenous Ckiδ was similar in lysates of cells transfected with either pcDNA-HA-CK1BP or vector alone (Figure 5.12A), indicating that transient expression of CK1BP did not alter levels of endogenous Ckiδ. Ckiδ was then immunoprecipitated from cell lysates using monoclonal antibody 128A and subjected to in vitro kinase assays using htau40 and [γ-32P]ATP as substrates. Radioactive reaction products were separated by SDS-PAGE and quantified by phosphoimager. Immunoblot analysis performed with monoclonal antibody 128A showed that the amount of Ckiδ in each immunoprecipitate was the same (Figure 5.12B). Moreover, the total amount of htau40 remaining after protein kinase assay was constant (Figure 5.12B). Nonetheless, Ckiδ immunoprecipitates prepared from cells transfected with pcDNA-CK1BP had significantly lower tau kinase than immunoprecipitates prepared from cells transfected with pcDNA vector alone (Figure 5.12BC). These results suggest that CK1BP is capable of modulating basal Ckiδ activity in HEK-293 cells as well as in vitro.
5.4 Discussion.

Owing to its natively unfolded structure and high complement of hydroxyamino acids, tau is an efficient substrate for many protein kinases in vitro (Ebneth et al., 1998). Here it was found that tau is a novel substrate for CK1 both in vitro and in situ. Overexpression of Ckiδ increased tau phosphorylation at sites it phosphorylated in vitro. However, the recognition sequence mediating the substrate selectivity of Ckiδ is not understood in the context of tau phosphorylation. On the basis of studies with short peptides, CK1 isoforms are phosphotropic kinases that recognize the motif S/T(P)XXS/T (Flotow et al., 1990; Flotow and Roach, 1991). Nonetheless, priming of substrates with phosphate is not a strict requirement, and motifs consisting of acidic residues N- or C-terminal to the phosphorylatable residue also are phosphorylated by CK1 (Marin et al., 2003; Marin et al., 1994; Pulgar et al., 1999). Although our in vitro data showing that Ckiδ phosphorylated recombinant tau at S\(^{202}/\)T\(^{205}\) and S\(^{396}/S^{404}\) is consistent with a previous study showing that tissue-derived CK1 activity phosphorylates tau at T\(^{231}\), S\(^{396}/S^{404}\) (Singh et al., 1996), the relationship between these sites and the motifs summarized above is weakly apparent only for S\(^{396}/S^{404}\). It appears that recognition of full-length tau as substrate differs from that of short peptides. In any event, phosphorylation of tau at S\(^{202}/S^{205}\) and S\(^{396}/S^{404}\) by Ckiδ does not require priming by other protein kinases.
CK1-mediated tau phosphorylation may have the potential to disrupt tau’s binding to microtubules, which will raise intracellular concentrations of free tau, and promote tau to form amorphous aggregation. CK1-mediated tau phosphorylation may also modulate tau turnover. This function may be part of the neuronal response to cellular stress.

CK1BP is a novel binding partner of the CK1 family in human brain. It and its putative longer variant derived by alternative splicing are paralogs of dysbindin, a ubiquitously expressed protein named for its ability to bind components of the dystrophin-associated protein complex (i.e., α- and β-dystrobrevins) in both muscle and nonmuscle cells (Benson et al., 2001). Nonetheless, little is known about the molecular activity of any dysbindin homolog beyond participation in macromolecular complexes in part because its protein domains are poorly characterized. Family members contain different combinations of up to three distinct segments: an N-terminal basic domain, a central coiled-coil domain, and a highly acidic C-terminal domain. Although the coiled-coil region functions to mediate complex formation with other coiled-coil containing binding partners (Benson et al., 2004) or potentially with itself, the function of the remaining protein segments has been unclear. Here we found that the acidic domain in isolation can bind CK1 isoforms. Because the acidic domains of both CK1BP and dysbindin bind truncation mutant Ckiδ-Δ317, and because the C-terminal extension of each CK1 isoform differs, it is likely that binding to intact CK1 isoforms is mediated primarily by their catalytic domains. CK1 catalytic domains contain abundant positive surface charge and are highly conserved among isoforms (Xu et al., 1995), and so electrostatic interactions may play a role in binding. Nonetheless, nonspecific charge-
charge interactions alone are insufficient for binding, because neither CK1BP nor the acidic domain of dysbindin bound Ckiα, despite it having a high isoelectric point and >75% identity with other CK1 isoforms.

On the basis of sequence alignments and the established crystal structures of fission yeast Cki1 and rabbit Ckiδ catalytic domains (Longenecker et al., 1996; Xu et al., 1995), the origin of this selectivity is not clear. None of the CK1BP or dysbindin variants contain docking motifs discovered in some CK1 substrate proteins, such as PER and NFAT (Okamura et al., 2004), beyond having clusters of acidic amino acid residues, and these are not preceded by SLS motifs as found in other CK1 substrates (Marin et al., 2003). The heat stability of CK1BP-mediated CK1 inhibition suggests that its interaction with CK1 isoforms is mediated mostly by primary structure. The Ckiα variant employed here may lack a small number of residues required to support high-affinity binding of CK1BP primary structure. Binding free energy in protein-protein interactions is not equally distributed at the binding interface. Rather, there are “hot spots” of binding energy consisting of a subset of residues at the interface (Clackson and Wells, 1995; DeLano, 2002). For example, TEM-1 and SHV-1 β-lactamases share 68% amino acid sequence identity and their atomic structures are nearly superimposable (Kuzin et al., 1999). But TEM-1 binds to β-lactamase inhibitor protein about 10,000-fold more efficiently. It has been suggested that the weaker binding of SHV-1 β-lactamases with inhibitor is due to only four residue changes in SHV-1 (Kuzin et al., 1999; Rudgers et al., 2001). This shows that small changes in “hot spot” residues may bring large changes in
binding specificity. In the case of CK1BP, “hot spot” residues may not exist in the Ckiα isoform. It is also possible that the short C-terminal extension of Ckiα, which differs among CK1 isoforms, interferes with binding.

CK1 binding activity also depends on the sequence context of the acidic domain. When isolated and in monomeric form, as exemplified by CK1BP, CK1 binding activity was readily observable. However, when presented in the context of dysbindin variant c, which contains a coiled-coil domain N-terminal to the acidic domain, CK1 binding activity was not detectable either in yeast using a two hybrid screen or in vitro using pull down assays. Binding activity was unmasked only after removal of the coiled-coil domain. This suggests that the coiled-coil domain interferes with the binding activity of the acidic domain either directly (by interacting with it) or perhaps indirectly (by fostering homodimerization or oligomerization). It is conceivable that conditions that disrupt the former, such as participation in higher order complexes (see below), or that disrupt coiled-coils, such as posttranslational modification (Steinmetz et al., 2001; Szilak et al., 1997), including proteolysis, may unmask CK1 binding activity in full-length dysbindin variants.

In addition to its ability to bind the dystrophin-associated protein complex, dysbindin is a component of BLOC-1 (biogenesis of lysosome-related organelles complex-1), a protein complex involved in the generation or trafficking of lysosome related organelles. These organelles are cell-type specific modifications of the post-Golgi membrane system that share various characteristics with lysosomes but that are specialized for storage and secretion rather than degradation. They include
melanosomes, platelet dense granules, lamellar bodies of type II alveolar epithelial cells, and lytic granules of cytotoxic T lymphocytes and natural killer cells, mast cell basophilic granules, Weibel Palade bodies of endothelial cells, azurophilic granules of neutrophils and eosinophils, osteoclast granules, and rennin granules of juxtaglomerular cells. Misfunction of dysbindin and other members of the BLOC-1 complex can lead to a syndrome (i.e., Hermansky-Pudlak Syndrome) characterized by deficient skin pigmentation (albinism), blood platelet dysfunction (prolonged bleeding), visual impairment, and abnormal storage of a fatty-like substance (ceroid lipofuscin) in various tissues of the body (Di Pietro and Dell'angelica, 2005). Lysosome/vacuole formation and function is linked to the secretory and endocytic pathways used in protein and lipid trafficking where CK1 is known to localize in postmitotic cells such as neurons (Gross et al., 1995). Moreover, some CK1 isoforms, such as Ckiδ, are major markers of granulovacuolar degeneration bodies found in AD and other neurodegenerative diseases (Ghoshal et al., 1999). In yeast, at least one CK1 isoform (Yck3p) resides in part with the vacuole (Sun et al., 2004) where it regulates budding and fusion reactions of this organelle (LaGrassa and Ungermann, 2005). Because CK1 frequently functions in tandem with the ubiquitin system, its potential interaction with dysbindin family members may be important for turnover and trafficking of protein complexes involved in formation of lysosome related organelles. Such a function would be specific to metazoans because unlike CK1, dysbindin homologs are not found in lower eukaryotes.

Whether CK1BP functions in secretory or endocytic pathways as part of macromolecular complexes like dysbindin remains to be determined. When free in
solution, CK1BP binding of CK1 results in inhibition of protein kinase activity. The IC$_{50}$ for this effect is ~60-90 nM for htau40 and α-synuclein (at 4 µM bulk concentration). However, IC$_{50}$s depend on the ratio of substrate concentration to $K_M$ (Cheng and Prusoff, 1973), and so higher CK1BP concentrations are required to inhibit the phosphorylation of efficient substrates such as casein. $K_i$ values, which do not depend on substrate concentration, are predicted to be significantly lower than estimated IC$_{50}$, and well within two orders of magnitude of the affinity of established protein kinase inhibitors including the cAMP-dependent protein kinase inhibitor (PKI; Ref. (Demaille et al., 1977)). The functions of inhibitors such as PKI are not entirely clear but may involve control of basal levels of protein kinase activity. Although some CK1 isoforms such as Ckiδ and ε are regulated in part via autophosphorylation of C-terminal domains (Cegielska et al., 1998; Graves and Roach, 1995), phosphotransferase activity remains constitutively active. Free CK1BP may function to inhibit basal CK1 activity in cytoplasm.

The yeast two hybrid system is a widely used genetic approach for detecting protein-protein interactions, accounting for almost 40% of identified interactions (Xenarios et al., 2002). The Sos recruitment paradigm employed here indicated that CK1BP was the only interacting species detectable by this method in a human brain library. Two prior screens employing Ckiδ as bait and GAL4 transcriptional activation assays identified scaffolding protein CG-NAP/AKAP450 and the light chain of microtubule associated protein 1A from human testis and bone marrow libraries, respectively (Sillibourne et al., 2002; Wolff et al., 2005). The emphasis on libraries from different mitotic cell types could account for differences observed between them and the
neuronal library employed here. For example, Cki₂ colocalizes with the spindle in mitotic cells (Behrend et al., 2000). But the microtubules of neuronal cells no longer organize their microtubules into spindles, instead forming more complicated structures that function in the context of cellular processes (axons and dendrites). Thus although CK1 isoforms are ancient and conserved among eukaryotes, it is not surprising that different binding partners would be discovered in different cell types. However, each of the GAL4-based screens generated additional positives beyond those reported and these may contain dysbindin/CK1BP family members. Because isolated full-length dysbindin does not bind CK1, two-hybrid screens employing full-length dysbindin as bait (Benson et al., 2004) would not be expected to identify any CK1 isoform unless accompanied by proteolysis or other post-translational alterations.

CK1 isoforms have been detected as prey in a number of two-hybrid screens using various proteins as bait including the SH2/SH3-domain containing adapter protein Nek (Lussier and Larose, 1997), metastatic tumor antigen 1 (Mishra et al., 2004), and components of Wnt signaling pathways (Kani et al., 2004). However, Cki₂ was not among the CK1 isoforms identified in these screens. These data are consistent with tight binding interactions among proteins being CK1 isoform selective.

Finally, binding partners for Ckiα have been sought by affinity chromatography, revealing various interacting partners, including protein phosphatase 1 inhibitor CPI-17, centaurin-α₁ and other proteins (Dubois et al., 2002; Dubois et al., 2001; Zemlickova et al., 2004). CK1BP does not bind Ckiα, and so should not appear in screens such as these.
5.5 Summary.

Casein kinase 1 is a family of ubiquitous eukaryotic protein kinases that frequently function in tandem with the ubiquitin modification system to modulate protein turnover and trafficking. In Alzheimer’s disease, these enzymes colocalize with ubiquitinated lesions including neurofibrillary tangles and granulovacuolar degeneration bodies, suggesting they also play a role in disease pathogenesis.

First, we characterized the contribution of one CK1 isoform, Ckiδ, to the phosphorylation of tau at residues S\textsuperscript{202}/T\textsuperscript{205} and S\textsuperscript{396}/S\textsuperscript{404} in human embryonic kidney-293 cells. Overexpression of Ckiδ increased CK1 enzyme activity and further raised tau phosphorylation at residues S\textsuperscript{202}/T\textsuperscript{205} and S\textsuperscript{396}/S\textsuperscript{404} in situ. These results suggest that Ckiδ phosphorylates tau \textit{in vivo}, and that the expression pattern of Ckiδ in Alzheimer’s disease is consistent with it playing an important role in tau aggregation.

Next, to identify binding partners that potentially regulate or recruit these enzymes toward disease lesions, a Sos-recruitment yeast two-hybrid screen was performed with human Ckiδ (the casein kinase-1 isoform most closely linked to granulovacuolar degeneration bodies) and a human brain cDNA library. All interacting clones contained a single open reading frame termed \textit{casein kinase-1 binding protein} (CK1BP). On the basis of sequence alignments, CK1BP was a structural homolog of the acidic domain of dysbindin, a component of the dystrophin-associated protein complex and the biogenesis of lysosome-related organelles complex-1. CK1BP interacted with full-length Ckiδ, the isolated Ckiδ catalytic domain, Ckiγ\textsubscript{2}, γ3, and ε in the yeast two-hybrid system, and bound Ckiδ and ε in pull down assays, but did not interact with Ckiα. Interaction with
the Ckiδ catalytic domain led to concentration-dependent inhibition of protein kinase activity in the presence of protein substrates tau and α-synuclein. Although intact dysbindin did not bind any CK1 isoform, deletion of its coiled-coil domain yielded a protein fragment that behaved much like CK1BP in two hybrid screens. These data suggest that the acidic domain of dysbindin and its paralogs in humans may function to recruit casein kinase-1 isoforms to protein complexes involved in multiple biological functions.
## 5.6 Tables

### CK1BP Physical Properties

<table>
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<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic radius (nm)</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>Sedimentation coefficient ($s_{20,w}$)</td>
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</tr>
<tr>
<td>Partial specific volume (mL/g)(^a)</td>
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</tr>
<tr>
<td>Native molecular mass (Da)(^b)</td>
<td>18,000 ± 1,000</td>
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<tr>
<td>Native structure</td>
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<tr>
<td>$f/f_o$(_{\text{obs}})</td>
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</tr>
<tr>
<td>$f/f_o$(_{\text{shape}})(^c)</td>
<td>1.47</td>
</tr>
<tr>
<td>Axial ratio (prolate ellipsoid)</td>
<td>8.7:1</td>
</tr>
</tbody>
</table>

\(^a\)Calculated from amino acid composition  
\(^b\)Estimated from hydrodynamic parameters  
\(^c\)Corrected for hydration assuming 0.5 g water/g protein

Table 5.1. CK1BP Physical Properties.
5.7 Figures.

**Figure 5.1.** Ckiδ phosphorylates tau at S202, T205, S396, and S404 *in vitro.*

Recombinant His$_6$-tagged htau40 (0.2 mg/ml) was incubated in the presence or absence of Ckiδ-Δ317 (50 ng) under phosphorylating conditions and subjected to SDS-PAGE and Western analysis. A, after 30 min at 37°C in the presence of [γ-$^32$P] ATP, phospho-tau could be detected by phosphorimaging. However, total tau as detected by Coomassie blue (CB) staining of SDS gels shows essentially no band shift. The bottom panel shows Ckiδ-Δ317 detected with monoclonal antibody 128A antibody. B, after 4 h at 37°C, total tau as detected with monoclonal antibody Tau5 shows a marked band shift. Phospho-tau species were detected by monoclonal antibodies PHF1 and AT8, suggesting that, at a minimum, Ckiδ-Δ317 phosphorylated sites S$^{202}$, T$^{205}$, S$^{396}$, and S$^{404}$ on htau40.
Figure 5.2. Ckiδ induces tau phosphorylation in situ.

HEK-293 cells transiently co-transfected (24 h) with p25/tau, Ckiδ/tau, and empty pcDNA vector/tau were harvested, lysed, and subjected to immunoblot analysis using monoclonal antibodies AT8, PHF1, Tau5, 128A, or C-19. A, cells transfected with empty vector showed measurable basal levels of phospho-tau and Ckiδ but not of p25. Transfection of cells with Ckiδ or cdk5 activator p25 significantly increased phospho-tau over basal levels. B, total tau (Tau5 epitope) and phospho-tau (PHF1 epitope) levels were quantified densitometrically from three individual experiments. When normalized for total tau content, phospho-tau was found to increase significantly with both p25 and Ckiδ transfection. *, $p < 0.01$; **, $p < 0.001$ when compared to empty vector control.
Figure 5.3. *In vitro* kinase assay.

The HEK-293 cells transiently transfected (24 h) with Ckiδ/tau or empty vector/tau were harvested, lysed, and subjected to immunoprecipitation assays using anti-Ckiδ antibody 128A. Ckiδ immunocomplexes from Ckiδ/tau (lane 1) or from empty vector/tau (lane 2) were then incubated *in vitro* with His6-htau40 and [γ-32P]ATP for 30 min and subjected to SDS-PAGE. *A*, phospho-tau detected by phosphorimaging shows that transfection with Ckiδ increased immunoprecipitable Ckiδ activity (32P), which correlated with levels of Ckiδ protein (128A). Bottom panel shows total tau detected by Coomassie blue staining. *B*, quantitative data from three independent experiments presented as mean ± S.E. **, *p* < 0.001 compared with empty vector control.
Figure 5.4. Identification of CK1BP.

(A), Frequency and location of 5’-ends of all positive clones (consisting of multiple copies of 11 unique sequences) relative to long (1.5 kB; accession number NM018478) and short (1.0 kB; accession number BC012818) transcripts associated with the open reading frame in the GenBank database (black bars denote coding sequences). All 5’ ends were consistent with the latter transcript being the source of Ckiδ interacting activity. The protein encoded by this transcript was named CK1BP. (B), Alignment of CK1BP amino acid sequence with C-terminal domain of dysbindin (DTNBP1 residue numbering corresponds to variant a). Identities are shown in black whereas similarities are shaded in gray. (C), Protein domains of CK1BP relative to dysbindin. Full-length dysbindins are derived from the DTNBP1 gene and consist of a weakly basic N-terminal domain (white bar), followed by a coiled-coil domain (striped bar), and finally a strongly acidic C-terminal segment (black bar). Dysbindin variant c lacks the N-terminal basic domain whereas variant b contains an alternatively spliced C-terminus. CK1BP shares homology with the strongly acidic, C-terminal domain of dysbindin variants (this domain is depicted with dark shading). Although the protein product of transcript NM018478 contains a weakly basic N-terminal segment, it is unrelated to the N-terminal sequence found in dysbindin and so is represented by a cross-hatch pattern. Numbered scale at bottom corresponds to dysbindin variant a.
Figure 5.5. CD spectra of CK1BP and His<sub>6</sub> tagged CK1BP.

(A), CK1BP (○) and His<sub>6</sub>-tagged CK1BP (●) were prepared in 100 mM sodium perchlorate, 20 mM boric acid, pH 7.4, and subjected to far-UV CD spectroscopy (25°C). (B), CD spectra were individually fit by CDSTTR, SELCON3, and CONTIN/LL algorithms to estimate total α-helical, β-strand, and random coil/turn conformation. The three estimates were then averaged and plotted ± SD for CK1BP (black bars) and His<sub>6</sub>-tagged CK1BP (white bars). Both preparations contained mostly disordered secondary structure, and did not differ significantly from each other at p < 0.05.
Figure 5.6. CK1BP selectively interacts with CK1 isoforms.

Cdc25Hα cells transformed with different combinations of bait and prey plasmids were grown under conditions that were non-selective (glucose minimal media at 25°C) or selective (galactose minimal media at 37°C) for interaction. Sos-V₀ and Myr-V₀ denote empty bait and prey vectors, respectively. CK1BP supported growth of Cdc25Hα cells in the presence of all CK1 isoforms examined except Ckiα. CK1BP also supported growth in the presence of Ckiδ-Δ317, indicating that interaction was mediated through the protein kinase catalytic domain or a short segment C-terminal to it.
Figure 5.7. CK1BP binds CK1 isoforms *in vitro*.

The ability of CK1 isoforms to bind His<sub>6</sub>-tagged CK1BP immobilized on Ni<sup>2+</sup>-NTA beads was assessed with pull-down assays performed as described in Experimental Procedures using HEK-293 cell lysate as the source of endogenous CK1 immunoreactivity. Ni<sup>2+</sup>-NTA beads alone or charged with His<sub>6</sub>-tagged α-synuclein were included to control for non-specific binding. Complex components were detected after beads were boiled and subjected to SDS-PAGE and immunoblot analysis. (A), Coomassie blue stain after SDS-PAGE showing presence or absence of input bait proteins. His<sub>6</sub>-tagged CK1BP but not α-synuclein migrates anomalously on SDS-PAGE. (B), immunoblot analysis performed using monoclonal antibodies raised against Ckiα (94.1), Ckiδ (128A), and Ckiε. Endogenous levels of all three of these CK1 isoforms were detectable in cell lysates. Both Ckiδ and Ckiε selectively bound immobilized His<sub>6</sub>-tagged CK1BP relative to immobilized His<sub>6</sub>-tagged α-synuclein or uncharged Ni<sup>2+</sup>-NTA beads alone, but Ckiα did not.
Figure 5.8. Dysbindin acidic domain selectively binds CK1 isoforms.

Cdc25Hα cells transformed with different combinations of bait and prey plasmids were grown under conditions that were non-selective (glucose minimal media at 25°C) or selective (galactose minimal media at 37°C) for interaction. The C-terminal acidic domain of dysbindin (C-term) composed of residues 184-351 of DTNB1 variant 1 (see Figure 1 for amino acid sequence) supported growth of Cdc25Hα cells in the presence of all CK1 isoforms examined except Ckiα, whereas full length dysbindin variant c (WT) did not.
Figure 5.9. CK1BP inhibits Ckiδ autophosphorylation.

HEK-293 lysates (25 µg total protein) were prepared and incubated in vitro (30 min at 37°C) with phosphorylation buffer in the presence or absence of 1 mM ATP and recombinant CK1BP (250 nM). Reaction products were separated by SDS-PAGE and detected on immunoblots with anti-Ckiδ antibody 128A. In the presence of nucleotide substrate, full-length Ckiδ underwent a band shift consistent with autophosphorylation. CK1BP inhibited substrate dependent band shift, consistent with an ability to antagonize autophosphorylation.
Figure 5.10. CK1BP inhibits Ckiδ-Δ317 activity in vitro.

Purified recombinant Ckiδ-Δ317 (34 nM) was incubated (30 min at 37°C) under phosphorylating conditions in the presence of 4 μM protein substrates, 100 μM [γ-32P]ATP, and varying concentrations of CK1BP (0 - 1024 nM). Reaction products were separated by SDS-PAGE, then fixed and stained with Coomassie blue (CB). Incorporation of 32P label was then analyzed by autoradiography. Increasing concentrations of CK1BP lead to a dose dependent inhibition of (A) His6-α-synuclein and (B) His6-htau40 phosphorylation. (C), Phospho-tau levels shown in Panel A were quantified by densitometry and plotted as percent inhibition (i.e., 0% inhibition corresponds to 32P incorporation in the absence of CK1BP). The line represents best fit of the data to a rectangular hyperbola (eq 1). CK1BP inhibition of Ckiδ-Δ317 mediated phosphorylation of His6-α-synuclein and His6-htau40 phosphorylation was saturable with IC50s of 57 ± 3 nM and 90 ± 8 nM, respectively.
Figure 5.11. CK1BP-mediated inhibition is heat stable.

Purified recombinant Ckiδ-Δ317 (34 nM) was incubated (30 min at 37°C) under phosphorylating conditions in the presence of 4 μM His₆-htau40, 100 μM [γ-³²P]ATP, and the presence or absence of 250 nM CK1BP (~3-fold above the IC₅₀). Reaction products were separated by SDS-PAGE, then fixed and stained with Coomassie blue (CB). Incorporation of ³²P label was then analyzed by autoradiography. Tau phosphorylation was inhibited by CK1BP, and this inhibition was retained even in the presence of heat-treated CK1BP (100°C for 3 min).
Figure 5.12. CK1BP inhibits Ckiδ activity *in situ.*

HEK-293 cells transiently transfected (48 h) with pcDNA-HA-CK1BP or empty vector were harvested, lysed, and subjected to immunoprecipitation with anti-Ckiδ antibody 128A. Immunocomplexes from each condition were then incubated *in vitro* (30 min) with protein kinase substrates His$_6$-htau40 and [γ-$^{32}$P]ATP. Reaction products were separated by SDS-PAGE and viewed in a phosphorimager. (A), immunoblot analysis performed on cell lysates with monoclonal antibodies 128A and 12CA5. Whereas levels of endogenous Ckiδ detected with monoclonal antibody 128A did not vary between the two conditions, HA-CK1BP was detected only in lysates prepared from cells transfected with pcDNA-HA-CK1BP. (B), Although levels of Ckiδ protein in the immunoprecipitates (detected with monoclonal antibody 128A) and of His$_6$-htau40 substrate (detected by Coomasie blue staining; CB) were indistinguishable, the amount of detectable phosphotau product ($^{32}$P) decreased in immunoprecipitates from cells expressing HA-CK1BP relative to empty vector control. (C), Quantitative data from three independent experiments presented as mean ± S.E. **, $p < 0.01$ compared with empty-vector control.
Figure 5.12. CK1BP inhibits Ckiδ activity in situ.
6 SUMMARY AND FUTURE DIRECTIONS

In Chapter 2 we showed that full-length tau proteins are refractory to aggregation because they must adopt assembly competent conformations before filament nucleation or elongation can proceed. Our data improved the understanding of tau fibrillization pathway which could not be explained sufficiently by classic nucleation-elongation theory, and suggested that triggers of tau aggregation were those conditions that could relieve the conformational barrier by stabilizing assembly competent conformations of tau. However, it is still not clear that whether different tau fibrillization inducers all work through this manner or not. Also, future research is desired to identify endogenous inducers in AD brain and to test whether this theory is also feasible in vivo.

We also established a convenient method for driving tau into assembly competent conformation using small-molecule inducers. They are anionic planar aromatic dyes known to bind β-sheet structure and used for stain AD lesions in situ such as Congo Red, thiazin red, and ThS. We then demonstrated the utility of this method for dissection of tau aggregation kinetics in vitro using C-terminal truncation mutants. Our data indicated that C-terminal truncations decreased critical concentration which reflected the increased fibrillization at reaction plateau. At the same time, truncations directly accelerated
nucleation rate, which made the whole aggregation procedure faster than wild type tau. Our work not only clarified the mechanism by which C-terminal truncation modulated tau aggregation, but also confirmed that advantage of using small molecular dyes as inducers to study tau aggregation mechanism in vitro. Comparing with previous inducers of anionic surfactants and microspheres, dyes induced tau aggregation in their monomer state, supported fibrillization at critical concentrations as low as 200 nM (well within the physiological range), and induced protein aggregation with near homogeneous nucleation kinetics. All of these will simplify data analysis and make experiment easier to proceed. Thus, in further studies, these dyes can also be used to clarify the assembly mechanisms of other post-translational modifications of tau protein, FTDP-17 mutations, and especially 3-repeat tau isoforms, whose fibrillization can not be efficiently induced by anionic surfactants in vitro.

Although our data indicated planar aromatic dyes as efficient inducers of tau fibrillization, it is still not clear about their binding sites on tau monomer. Next, since the concentration response curves were biphasic, it will be interesting to test whether high concentration of dyes can inhibit tau fibrillization in the present of other inducers such as anionic surfactants and microspheres. In addition, further study on the structural characteristics of these dyes may also helpful for the future design of inducer compounds.

In Chapter 4, we showed the feasibility of this method for driving tau aggregation in living cells. Congo Red was used to treat the tau stable cell line. 10 µM Congo red efficiently induced detergent insoluble aggregates after 7 days incubation, with approximately 30% of total tau went into aggregates. Tau aggregates lost its tubulin binding activity, which is independent of tau phosphorylation. On the basis of viable cell
counts, aggregation was not toxic up to 14 days in culture. However, the presence of proteasome inhibitor ALLN unmasked a tau-aggregate dependent loss of viability. Overall, the data addressed the issue of tau fibrillization as a mediator of cell death in tauopathic neurodegenerative diseases. Our future plan is to establish tau aggregation model in primary neuron cultures with Congo red as inducer. Tau is endogenous in neurons. Thus not only we can test whether Congo red is capable to induce tau aggregation in its physiological level, but also it will be easier to examine the toxicity of tau aggregation without the disturbance by the toxicity resulting from tau overexpression. It will be more informative to further examine whether tau aggregations will result in loss of axonal transport, cytoskeletal disorganization, and neuron death.

In the above chapters, we studied the conditions that trigger tau fibrillization, the role of c-terminal truncations of tau and established a cell model of tauopathy without referring to tau phosphorylation, another critical post-translational modification that regulates the functions of tau protein. Because AD-derived tau protein is hyperphosphorylated relative to protein isolated from non-demented control tissue, and aberration in its phosphorylation state may modulate tau fibrillization and neuritic lesion formation which occur in Alzheimer's disease and other neurodegenerative conditions, it is very important to identify the protein kinases or signaling cascades that regulate tau phosphorylation in vivo. These objectives led to the works described in Chapter 5.

In the first part of Chapter 5, a member of the CK1 family of protein kinases was tested for its ability to phosphorylate tau protein because previous data showed that CK1 highly over-expressed in AD tissue, suggesting they may be involved in tau hyperphosphorylation and neuritic lesion formation. Our results demonstrated that Ckiδ
phosphorylated tau both *in vivo* and *in vitro*; the up-regulation of Ckiδ observed in authentic AD tissue may contribute to tau hyperphosphorylation. Although we confirmed that Ckiδ phosphorylated tau at residues Ser202/Thr205 and Ser396/Ser404 *in situ* with antibodies phospho-tau specific antibodies AT8 and PHF1, it is possible that residues phosphorylated by Ckiδ are more than what we examined in this current work. Further investigations on phosphorylation sites will be beneficial if we try other phosphorylation site-specific antibodies such as those detect phospho-tau-S\(^{199}\), phospho-tau-T\(^{231}\), and phospho-tau-S\(^{262}\). Mass Spectrometry/Mass Spectrometry could also be used to accomplish this purpose. In addition, to further investigate the function of Ckiδ in AD, similar experiment can be performed in primary neuron cultures. Moreover, IC261, the specific inhibitor of CK1, can be intraventricular infused to a transgenic model of AD to examine the changes in tau hyperphosphorylation, filament formation, cytoskeleton integrity, and neuron death.

From above data, we got more information on the function of CK1 in neurodegeneration diseases. Although CK1 is ubiquitous in all eukaryotes and has been implicated in a great number of physical functions, the mechanisms underlying their regulation are unknown. To indentify proteins that regulate CK1, which may lead to the finding of signaling cascades that regulate tau phosphorylation *in vivo*, we initiated a search for specific binding partners using a two-hybrid screen in yeast. Experimentation began with human Ckiδ (the CK1-isoform most upregulated in AD) as bait to probe a human brain cDNA library. The screen identified 18 positive clones, all of which encoded to a single potential binding partner of 161 amino acids termed CK1BP. We also
described the purification and characterization of CK1BP. The secondary structure of CKBP was examined by circular dichroism, which showed that CK1BP is predominantly unordered conformation and only a small fraction of the peptide exists in ordered conformation, mostly α-helical structure. His₆ tag did not bring significant change in the secondary structure of CK1BP. Size exclusion high-performance liquid chromatography and sucrose gradient sedimentation analysis proved that CK1BP are monomeric and highly asymmetric molecules in solution. CK1BP interacted with Ckiδ, Ckiδ-Δ317, Ckiγ2, γ3, and ε in the yeast two-hybrid system, and bound Ckiδ and ε in a His fusion pull down assay, but did not display interaction with Ckiα. Interaction with the Ckiδ catalytic domain led to concentration-dependent inhibition of protein kinase activity in the presence of protein substrates tau and alpha-synuclein. Dysbindin, CK1BP’s structural homologue protein, did not bind any CK1 isoform. But dysbindin acidic domain behaved much like CK1BP and selectively bound CK1 isoforms. Thus casein kinase-1 isoforms may be recruited to protein complexes though the acidic domain of dysbindin, and functions in dysbindin associated diseases such as muscular dystrophy, Hermansky-Pudlak syndrome and schizophrenia. Despite the above achievements, there are still many questions left behind. First, future studies are needed to examine the tissue distribution of CK1BP. The tissue-specific RNA and protein level of CK1BP can be quantified by Northern and by Western blot respectively. Similarly, we may also inspect its subcellular localization. Also, it is important to test whether CK1BP and CK1 directly bind each other in authentic tissues. To fulfill all of these objectives, generation of CK1BP antibodies is essential. Second, mapping and studying the minimum binding
motifs of CK1BP will help future design of CK1 inhibitors. Third, since our results showed that CK1BP interacted with Ckiδ catalytic domain and it is also a weak substrate of this kinase, thus it is ideal to know whether the interaction is phosphorylation-dependent. Two inactive mutations of Ckiδ (K38M and T176I) could be used to test whether they can still bind CK1BP. Or this question can be answered by testing the bindings between Ckiδ and mutated CK1BP whose phosphorylation sites of Ckiδ were all mutated by site-directed mutagenesis. Last and the most important future directions are to decipher the physiological functions of CK1BP. Since CK1BP is a novel protein, little is known about its functions except that it is the binding partner of Ckiδ. Without enough references, it is really hard to analyze its functions. To get some preliminary information, we can either overexpress CK1BP or knock it out using antisense oligomers and RNAi in its native cell lines, and then analyze changes of characteristics occurred with these manipulations.


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