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Review

Phosphorylated tau and the neurodegenerative foldopathies

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Abstract

Many studies have implicated phosphorylated tau in the Alzheimer disease process. However, the cellular fate of phosphorylated tau has only recently been described. Recent work has shown that tau phosphorylation at substrate sites for the kinases Cdk5 and GSK3-beta can trigger the binding of tau to the chaperones Hsc70 and Hsp27. The binding of phosphorylated tau to Hsc70 implied that the complex may be a substrate for the E3 ligase CHIP and this possibility was experimentally verified. The presence of this system in cells suggests that phosphorylated tau may hold toxic dangers for cell viability, and the response of the cell is to harness a variety of protective mechanisms. These include binding to chaperones, which may prevent more toxic conformations of the protein, ubiquitination which will direct the protein to the proteasome, segregation of tau aggregates from the cellular machinery, and recruitment of Hsp27 which will confer anti-apoptotic properties to the cell.

Keywords: Hsc70; Hsp27; CHIP; Cdk5 and GSK3-beta; Phosphorylated tau

1. Introduction

Nearly all of the neurodegenerative disorders can be broadly viewed as disorders of protein folding and the consequences of misfolding. In the broadest terms, these diseases might be classified as foldopathies. Within this disease category, accumulations of misfolded proteins may deposit intra- or extracellularly. The mechanism by which the cell attempts to clear accumulations distinguishes the pathological features of these two sites of deposition. Thus, an increasingly emerging theme among the neurodegenerative disorders with intracellular inclusions is a contributory defect in pathways that lead to effective protein degradation. The role of degradative and folding pathways is probably relevant for the extracellular foldopathies as well, but data on this topic are scarce [1]. Most well known among these extracellular depositions is Alzheimer's disease and congophilic angiopathy, in which the A-beta peptide deposits in plaques or in the walls of cerebral blood vessels. Among these diseases are the many amyloidoses including senile systemic amyloidosis and familial amyloid polyneuropathy due to transthyretin, light-chain amyloid due to immunoglobulin light chains, dialysis-related amyloid due to beta2 microglobulin, amyloidosis due to apolipoprotein A1, familial amyloidosis of Finnish type due to mutated gelsolin, prion diseases, hereditary cerebral hemorrhage with amyloidosis of the Icelandic type due to mutant cystatin, familial British dementia and familial Danish dementia, (reviewed in Refs. [2,3]. CADASIL in which Notch 3 deposits in blood vessel walls is not strictly an amyloidosis, but probably belongs to the extracellular foldopathies [4].

This review will focus on the degradation of tau, one of the most common intracellular inclusions. However, all of the pathological inclusions are mutually informative and data on other intracellular inclusions will be discussed here where relevant. Apparent from comparing available data among the intracellular foldopathies is that each of these inclusions has a distinct interface with the degradation and

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folding machinery and therefore the detailed mechanisms differ for each inclusion.

In Alzheimer's disease, tau deposits form paired helical filaments (PHFs) and/or straight filaments (SFs). In tauopathies caused by tau mutations, tau filaments can form twisted ribbons or rope-like filaments, and are located not only in neurons, but also in glia. A protein degradation defect very likely contributes to the failure to remove tau inclusions, as suggested by the presence of polyubiquitinated tau in the neurofibrillary tangles (NFTs). The finding of tau mutations in frontotemporal dementia provides a cogent basis for thinking of tau protein as the initiator of a pathogenic cascade related to protein degradation pathways and leading to the inclusions. Mechanisms for tau clearance must be inoperative or overloaded in affected cells.

Pinpointing exactly the degradative defect and positioning this defect in the disease cascade is the challenge. The first step is to define the toxic moiety. In the case of tau, the toxic moiety may be phosphorylated tau, a proteolytic fragment of tau, tau protofibrils or the assembled filaments. For each of these possibilities, defining the toxic moiety requires further precision. For example, if phosphorylation is culpable, then precisely which phosphorylation sites lead to disease? Inseparable from identifying the toxic moiety is revealing the mechanism of the toxicity. In the context of this review, the possibilities range from mechanisms related to degradative failure and build-up of toxic products versus direct toxicity of a form of tau and the recruitment of degradative mechanisms to prevent toxicity. For example, ubiquitination may enhance the toxicity of tau and lead to aggregation; alternatively, the addition of ubiquitin may diminish the toxicity and sequester otherwise toxic intermediates as inclusions.

2. Lessons from other neurodegenerations

Other neurodegenerative entities with ubiquitinated inclusions may be instructive for understanding the underlying pathogenesis of the tauopathies. In postmortem patient material as well as several mouse and fly models of polyglutamine disease, mutant proteins aggregate and are immunoreactive for ubiquitin, heat shock proteins, and components of the proteasome [5]. It is commonly believed that the polyglutamine expansion alters the conformation of the native protein so that it resists degradation. Recently, Steffan et al. [6] showed that SUMO modification of huntingtin inhibits its ubiquitination for proteasomal degradation. This observation links the ubiquitin–proteasomal system to the pathogenesis of Huntington disease. Partitioning an abnormally folded protein into an aggregate may actually prevent neurotoxicity. Among the lines of support for this hypothesis is the protection of neurons with the overexpression of chaperones. Overexpression of heat shock proteins in both flies and mice increases resistance to polyglutamine-induced toxicity. In SCA3 flies with 78Q, overexpression of HSP70 suppresses the phenotype and improves survival compared to controls with a dominant-negative HSP70 mutant [7]. Similarly, SCA1 mice with 82Q that overexpress HSP70 chaperone have significantly improved motor control and Purkinje cell morphology compared to their SCA1 littermates [8]. On the other hand, inactivation of ubiquitin, ubiquitin carrier enzymes, or a ubiquitin C-terminal hydrolase in an SCA1 fly model all worsened the neurodegenerative phenotype, and overexpression of a DNAJ1 chaperone abrogated the phenotype [9]. Furthermore, the loss of function of the ubiquitin ligase Ube3a in SCA1 mice increased neurodegeneration while decreasing the number of nuclear aggregates [10]. Knock-in SCA1 mice form aggregates in the most vulnerable cells last and cells which are spared form aggregates early on [11]. In these models, it appears that sequestering of the aggregate can curtail their toxicity. Instead toxicity may arise from smaller soluble protein assemblies that may exist as oligomers or protofibrils.

However, not all the data support a protective role for the inclusions in polyglutamine diseases. Toxicity may be directly caused by the abnormally assembled protein or due to recruitment of factors essential for cell survival by the abnormal protein. Expanded polyglutamine proteins sequester the nuclear receptor co-repressor (N-CoR) and CREB-binding protein (CBP) in mouse models and may cause detrimental effects on transcription [12–17]. Similarly, entrapment of Bcl-2 by large SOD1 aggregates in amyotrophic lateral sclerosis may deplete motor neurons of this anti-apoptotic protein [18]. Mechanisms that ordinarily enhance cell survival and fail in neurodegeneration include the protection that Hsp70 provides by preventing activation of stress kinases that lead to cell death [19], blocking procaspase processing [20] or caspase activation [21]. By binding to misfolded proteins, Hsps may prevent their interaction with other factors.

In Parkinson disease affected neurons contain intracytoplasmic protein aggregates called Lewy bodies and Lewy neurites, which contain alpha-synuclein [22,23], synphilin-1 (reviewed in REF. [24]), ubiquitin [25,26], parkin (an E3 ubiquitin ligase) [27], and in about 1–5% of cases, Hsp70-positive immunoreactivity [28]. Among the rare familial forms of Parkinson disease, the most common mutations occur in *parkin*. These are loss of function mutations that impair the ability of Parkin to ubiquitinate an *O*-glycosylated isoform of alpha-synuclein [29] as well as the synaptic-vesicle-associated protein CDCrel-1 [30], PAEL-R [31] and synphilin-1 [32]. Parkin expression may also be linked to the removal of misfolded proteins after cellular stress as its expression increases with cellular stress and it can suppress cell death associated with stress [31]. Another locus involved in inherited forms of Parkinson's disease, known as PARK5, encodes the enzyme UCHL1 [33], which is also involved in the ubiquitin–proteasome pathway. Similar to the polyglutamine disease models described above, Hsp70 can also modify alpha-synuclein

toxicity. In *Drosophila*, overexpression of Hsp70 inhibits dopaminergic neuronal loss induced by alpha-synuclein overexpression and, conversely, reduced Hsp70 activity enhances alpha-synuclein toxicity [28].

Data on alpha-synuclein may bear directly on tau inclusions because these two proteins share many features. Both tau and alpha-synuclein are heat-stable cytosolic proteins with extended structures that are capable of forming intra-neuronal inclusions. The normal proteins are both enriched in neuronal cytoplasm. Pathologically, tau and synuclein inclusions overlap in their disease phenotypes. Alzheimer's disease has a Lewy body variant, frontotemporal dementia has a Parkinsonian phenotype, and a tau haplotype has been associated with Parkinson's disease [34]. The tau haplotype associated with Parkinson's disease is more efficient in driving tau transcription [35]. Under pathological conditions, both tau and alpha-synuclein can accumulate in the same cell [36] and even co-assemble [37]. Both missense and splice site mutations in the tau gene are responsible for a phenotypically diverse group of hereditary neurodegenerative diseases, called frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) [38–40]. These tauopathies appear to involve misfolding and a degradative failure.

Increased phosphorylation of tau has been a persistent and longstanding theme among the hypotheses that could explain the conversion of normal tau to NFTs. Work on alpha-synuclein has similarly implicated hyperphosphorylation as a possible trigger in the conversion to Lewy bodies or a step toward the acquisition of toxicity [41]. In both cases, the extent of ubiquitination at any single site is usually below four [42,43], the number required for ubiquitin-dependent degradation [44].

3. Protein folding, degradation pathways, and cellular inclusions

Proteolysis is a key mechanism for maintaining the intracellular environment relatively free of misfolded proteins [45] and for the most part this function is carried out predominantly by the ubiquitin/proteasome system [46,47]. The covalent ligation of multiple ubiquitin molecules signals to the 26S proteasome, a multi-subunit complex in which two 19S regulatory 'cap' structures sit at each end of the 20S barrel-shaped chamber. The 19S 'caps' mediate the recognition of polyubiquitin as well as substrate unfolding through the action of a ring of ATPase molecules adjacent to the 20S chamber. The unfolded substrate is then threaded into the inner chamber of the 20S sub-complex and cleaved (reviewed in Refs. [46,48,49]).

Often the pathway to the proteasome requires a collaboration between the ubiquitin–proteasome and chaperone systems. Indeed, a significant proportion of newly synthesized proteins are either chaperone-associated [50] or rapidly degraded via the ubiquitin–proteasome pathway [51]. As first shown with firefly luciferase [52] and subsequently for other proteins including some involved in neurodegeneration, mammalian Hsp90, in cooperation with Hsp70, p60, and other factors, mediates the ATP-dependent refolding of heat-denatured proteins and failure to refold results in proteolysis. Thus, manipulating the chaperone proteins can alter the balance between correct folding and degradation of chaperone substrates.

Dysfunction in this system is believed to contribute to a variety of human diseases [53–57]. For many of the intracellular foldopathies, the implication of degradative systems is indirect and generally consists of a ubiquitinated inclusion, sometimes in complex with a chaperone, and the plainly evident fact that the inclusions failed to clear [58,59]. In the case of polyglutamine repeats, protein aggregates cannot be easily degraded [60] and their toxicity is enhanced in haploid yeast mutants when a group of genes functionally related to stress, protein folding, and ubiquitin-dependent protein catabolism were deficient [61]. In a few cases, the evidence favoring a degradative pathway in disease pathogenesis is more direct. For example, loss of function mutations of the *parkin* gene, which encodes an E3 ligase, leads to Parkinson's disease [62].

Primary defects in protein folding and degradative machinery have not been described in the tauopathies. What is known is that mutations in tau can lead to inclusions. This fact leads to several heuristic questions: at what point in a cascade that begins with mutant tau is there an interface with the folding and degradative machinery? How does this machinery either worsen or ameliorate the inclusions and ultimately the toxicity? The covalent linkage of tau to ubiquitin in the tau inclusions represents an incontrovertible starting point. Ubiquitination of tau has been detected in PHF fractions (reviewed in Ref. [63]). Both mono- and polyubiquitinated forms were detected by mass spectroscopy. The implications of mono- and polyubiquitination are functionally distinct with only polyubiquitination capable of targeting proteins to the proteasome. Ubiquitination has often been considered a "late" feature of the pathology because ubiquitinated tau was detected in the insoluble PHF fraction. However, more recent studies have observed the early appearance of ubiquitin in association with neurofibrillary pathology [64]. Curiously, a recent report also describes a family with frontotemporal dementia linked to chromosome 17q21–22 (locus of the tau gene) without a tau mutation and without tau inclusions, but with ubiquitin-positive inclusions [65]. Given the role of protein degradation in other neurodegenerative disorders, the role of tau ubiquitination requires revisiting.

4. Tau pathobiology

The carboxyl end of tau encodes three or four imperfectly repeated sequences, each capable of binding to microtubules [66,67]. Whether three or four repeats are expressed

depends on the alternative splicing of exon 10, a cassette exon that encodes the second repeat. The splicing of exon 10 (and the other alternatively spliced exons as well) is under complex temporal (developmental) and spatial (anatomical) regulation. The inclusion of this 93 nucleotide cassette exon, as well as the other two alternatively spliced exons (exons 2 and 3) expressed in the CNS, occurs relatively late in the course of brain development at about postnatal day 7 in the rat [68]. Once tau undergoes alternative splicing, isoforms of tau that include and exclude exon 10 are expressed in the human [69] and the presence of exon 10 increases the affinity of tau for the microtubule.

Flanking the repeats is a region of tau that is phosphorylated by multiple different kinases and is thought to confer fine regulation of microtubule binding [70]. Up to 19 different sites can be phosphorylated on tau [71], thus creating a staggeringly high number of possible tau phospho-isoforms. Little is known about the function of these different phosphorylation states other than that they alter the binding kinetics of tau to microtubules. Not only are many different sites phosphorylated on tau allowing for quite subtle regulation of microtubule binding, but also the control over the sites can be regulated through different signaling pathways as indicated by the many different kinases capable of phosphorylating tau. Among the kinases capable of phosphorylating tau in vitro are both proline-directed kinases and non-proline-directed kinases. They include glycogen synthase kinase 3beta (GSK3beta), extracellular signal regulated kinase (ERK), stress-activated protein (SAP) kinase, the cyclin-dependent kinase 5 (Cdk5), CDC2-cyclin A kinase, MARK kinase, Ca^{2+}/calmodulin-dependent protein kinase, cyclic AMP-dependent protein kinase (PKA), protein kinase C, casein kinase I and II, double-stranded DNA-dependent protein kinase, MAP/microtubule affinity-regulating kinase (MARK), and tau-tubulin kinase (reviewed in Ref. [72]). In culture, the expression of key kinases that phosphorylate tau changes over time [73]. Important changes in expression continue to occur even between 3 and 4 weeks in culture.

Because tau in PHFs is highly phosphorylated, phosphorylated tau has been viewed suspiciously as the toxic moiety. Much evidence has been garnered to support this view. In flies, increased hyperphosphorylated tau can cause neuronal cell death [74]. Fath et al. [75] showed that replacement of certain amino acids at known sites of phosphorylation with a charged amino acid to create 'pseudohyperphosphorylated' tau can mimic structural and functional aspects of hyperphosphorylated tau: in differentiated PC12 cells, PHF-tau exhibited reduced microtubule interaction and caused apoptotic cell death.

Another approach to pursuing the hypothesis that phosphorylated is the toxic moiety is to manipulate the tau kinases. In vivo evidence for an interaction with tau exists for Cdk-5 and GSK-3beta. Noble et al. [76] crossed transgenic mice overexpressing the Cdk5 activator p25 with transgenic mice overexpressing mutant (P301L) human tau. Tau was hyperphosphorylated at several sites in the double transgenics, and a highly significant accumulation of aggregated tau occurred in the brainstem and cortex. Increased numbers of silver-stained NFTs accompanied these changes as well as an association of active GSK3-beta with insoluble tau. Cruz et al. [77] showed that mice transgenic for p25, the truncated cdk5 activator, exhibited neuronal loss in the cortex and hippocampus, accompanied by forebrain atrophy, astrogliosis, and caspase-3 activation. Moreover, endogenous tau was hyperphosphorylated, aggregated tau accumulated, and neurofibrillary pathology developed progressively in these animals. The importance of dephosphorylation in neurofibrillary disease is highlighted by the ability of the phosphorylation-dependent prolyl isomerase, Pin1, to provide relative protection from age-dependent neurodegeneration [78]. Pin1 recognizes specific phosphorylated serine or thorenine residues in tau that are followed by a proline and catalyzes a critical conformational change that allows dephosphorylation at these residues. PP2A and PP1A are the major phosphatases of the phosphorylated tau [79]. PP2A is very abundant in brain and is associated with microtubules [80]. Sun et al. [81] showed that inhibition of protein phosphatase 2A and protein phosphatase 1 by calyculin A induced tau hyperphosphorylation and impairment of spatial memory retention in rats. Gong et al. [82] showed that the PP2A inhibition by okadaic acid resulted in increased phosphorylated tau in the axons in metabolically active rat brain slices. Overexpression of GSK-3β under the control of a tetracycline-sensitive transactivator also induced tau hyperphosphorylation, somatodendritic mislocalization of tau, and neuronal apoptosis [83]. However, the complexity of the role of GSK-3beta was underscored in transgenic mice that expressed a constitutively active mutant form of human GSK-3beta and surprisingly did not show neurofibrillary pathology. In fact, when crossed with the htau40 transgenic mouse, they improved the axonal dilations and motoric problems observed in htau40 mice [84]. Nevertheless, most of these studies implicate tau hyperphosophorylation in tau-related neurodegeneration.

Because overexpression of either a kinase or the substrate, i.e., tau, can lead to neurofibrillary pathology, multiple degrees of freedom exist over the circuitry that leads to pathology. The multiple limbs of the pathways that control tau phosphorylation further complicate predictions of regarding changes in the activity of key regulatory elements. For example, increasing the activity of protein phosphatase 2A could dephosphorylate tau, but it would also activate the GSK3-beta and potentially increase tau phosphorylation. What would happen with this manipulation in vivo is unknown, but a counter intuitive result is among the possibilities.

Knowledge of tau biology and the characterization of human tau mutations have opened the way to animal models of the tauopathies; however, convincing cell culture models remain elusive. The absence of robust culture models

remains a major lacune in tauopathy research. Overexpression of tau in either neurons or in heterologous cells does not usually produce readily detectable inclusions. This problem is surprising because several transgenic mouse models exist [85]. Furthermore, PHF-like deposits can be induced in lamprey reticulospinal neurons after massive overexpresssion of wild-type tau [86]. Transgenic flies expressing wild-type or the FTDP-17 mutations R406W or V337M show late-onset disease, accumulation of mutant tau, progressive degeneration, shortened life span, selective neuronal vulnerability, but no detectable NFTs [87]. The dissociation between the toxicity and inclusions has led to the hypothesis that large aggregates are not the principal cause of pathogenesis, instead protofibrils or small aggregates may be responsible for disease. Whether inclusions are a sine qua non for tau pathology is intensely debated. In vitro large polymeric aggregates can form from non-phosphorylated bacterially synthesized tau [88], and if these aggregates are necessary for human disease, then the importance of phosphorylation to the pathology is diminished. On the other hand, if tau phosphorylation creates the toxic entity prior to filament assembly then inclusions may be unnecessary for disease.

5. Tau degradation pathways: search for a tau E3 ligase

Ubiquitination is a cellular process by which short-lived or damaged proteins are conjugated with multimers of Ub, marking them for degradation in the proteasome. Conjugation requires an enzymatic cascade system that includes E1 Ub activating enzyme, E2 Ub conjugating enzyme (Ubc), and E3 Ub ligase enzyme. Tau is known to be conjugated to ubiquitin, a finding that implies the existence of an E3 ligase, which will mediate the conjugation. We reasoned that a tau E3 ligase might remain in association with Alzheimer NFTs. Cbl, a Ub ligase, selectively conjugates Ub onto tyrosine phosphorylated target molecules such as platelet derived growth factor receptor, epidermal growth factor receptor, Syk, and Fyn [89]. We therefore hypothesized that hyperphosphorylated tau could be ubiquitinated by an unknown E3 Ub ligase(s) for proteasomal delivery and degradation. The experiments reviewed below support the view that CHIP/Hsc70 complex selectively ubiquitinates phosphorylated tau in collaboration with UbcH5B [90]. Furthermore, CHIP attenuates phosphorylated tau-induced cell death.

First, we sought to compare in vitro ubiquitination of tau from Alzheimer frontal cortex with tau from control frontal cortex. We extracted and immunoprecipitated tau with an antibody cocktail that included 5E2 [91,92] and affinity purified polyclonal tau antibodies. The extraction utilized 1% Triton X-100 and therefore the more insoluble forms of tau associated PHFs would not be solubilized. After elution from the tau antibodies, we carried out in vitro ubiquitination with commercially available mammalian E1, E2 fractions and FLAG-Ub. FLAG-Ub conjugated molecules were immunoprecipitated with anti-FLAG antibody beads and probed with tau antibodies. Interestingly, Alzheimer tau was ubiquitinated and control tau was not. If the Alzheimer tau was washed in 1 M NaCl before ubiquitination, it was not ubiquitinated in vitro. This indicated that a 'co-factor fraction,' which washed off the Alzheimer tau, contained an E3 Ub ligase for tau.

These in vitro ubiquitination reactions utilized commercially available E1 and E2 fractions. With single specific E2s in the in vitro ubiquitination reaction, we found that Alzheimer tau was intensely and consistently ubiquitinated with UbcH5B. Other E2s tried in the reaction mixture did not ubiquitinate Alzheimer tau. This result strongly suggested that the E2 conjugating enzyme for tau ubiquitination is UbcH5B.

The ubiquitination of Alzheimer tau implied that normal tau may be degraded in a ubiquitin-independent pathway as is alpha-synuclein 88, [93–96], and led to the question, what modifications to Alzheimer tau are recognized by the E3 Ub ligase? The most likely modifications are phosphorylation and/or glycosylation. Our experiments showed that an Alzheimer type phosphorylation of tau served as the signal for in vitro tau ubiquitination. Alzheimer tau was immunoprecipitated from brain and treated with protein phosphatase 2A (PP2A), protein tyrosine phosphatase 1B (PTP1B), *N*-glycosidase, sialidase or *O*-glycosidase. Only PP2A treatment prevented in vitro ubiquitination. These data indicated that phosphorylation of PP2A dephosphorylation sites is an important recognition signal for ubiquitination. Other data are consistent with these findings. PP2A is very abundant in brain and is associated with microtubules [97]. By using metabolically competent rat brain slices as a model, selective inhibition of PP2A by okadaic acid induced an AD-like hyperphosphorylation and accumulation of tau [98]. Many of the sites that are dephosphorylated by PP2A are phosphorylated by either GSK-3beta or Cdk5. These include S199, S202, T205, S396, and S404 93-96.

We phosphorylated His tagged full-length recombinant human tau with GSK-3beta in vitro. The phosphorylated tau protein reacted on immunoblots with PHF1 [99] and AT8 [100], indicating that at least sites S202, T205, S396, and S404 were phosphorylated. This in vitro phosphorylated tau served as an excellent substrate for in vitro ubiquitination using UbcH5B and the co-factor fraction (see above) from Alzheimer tau immunoprecipitates. This finding suggested that GSK-3beta can place phosphates on tau that create recognition sites for an E3 Ub ligase. Cdk5 was also capable of phosphorylating tau in a manner that allowed recognition by an E3 Ub ligase.

The in vitro phosphorylated recombinant tau was used to find a specific E3 ligase capable of ubiquitinating tau. In vitro phosphorylated recombinant tau was bound to a column and normal human brain extract was passed over the column. The bound fraction was eluted and passed over a second column bound to amino-terminal GST-UbcH5B. The fraction bound to this column retained E3 Ub ligase

activity for the recombinant phosphorylated tau substrate. The sample contained five bands by Coomassie brilliant blue (CBB) staining (from 27 to 70 kDa). A mass spectroscopy analysis of the 70-kDa band revealed that it corresponded to the heat shock cognate 70-kDa protein (Hsc70). The 27-kDa band corresponded to Hsp27 [90], which we will discuss further below. Also present (Shimura and Kosik, unpublished data) was dihydropyrimidinase-related protein 2 (DRP-2), known as collapsin, which is involved in axonal growth and guidance and is a component of PHF. DRP-2 is a target of protein oxidation in the AD brain [101]. The remaining band detected corresponded to elongation factor EF-1 alpha. Protein synthesis elongation factor EF-1 alpha is essential for ubiquitin-dependent degradation of certain *N*-alpha-acetylated proteins [102]. These authors proposed that EF-1 alpha may be involved in releasing ubiquitin from multiubiquitin chains, thus rendering the conjugates susceptible to the action of the 26S protease complex. We also identified excitatory amino acid transporter EAAT-2 as part of a complex in association with hyperphosphorylated tau (Shimura et al., unpublished observation). Previous studies showed that EAAT-2 localized to tangle-bearing neurons in Alzheimer's disease [103].

Initially, we focused on Hsc70. In contrast to the phosphorylated tau, non-phosphorylated tau did not bind Hsc70. The presence of Hsc70 in the complex suggested a degradation pathway that utilizes CHIP (carboxyl terminus of the Hsc70-interacting protein) as the E3 ligase [104]. The co-chaperone protein, CHIP, has three tetratricopeptide repeats that bind to Hsc70/Hsp70 and Hsp90, while its C-terminal U-box domain associates with E2, thus satisfying the requirements of a chaperone-associated Ub ligase. Several proteins, such as the cystic fibrosis transmembrane conductance regulator [105], ErbB2 [106], the glucocorticoid receptor and c-raf kinase [107], were identified as target molecules of CHIP. Murata et al. [108] reported that heat-denatured luciferase was ubiquitinated by CHIP, and proposed that it selectively ubiquitinates unfolded protein(s) by collaborating with molecular chaperones. Therefore, CHIP appears to play a role in targeting chaperone–protein complexes to the proteasome. In our experiments, the same fraction that contained Hsc70 also contained an immunoreactive band at the correct molecular weight of CHIP. We also eluted exogenously expressed myc-CHIP from affinity phosphorylated His-tau column and GST-UbcH5B column. Furthermore, recombinant GST-CHIP ubiquitinates phosphorylated tau in vitro in the presence of UbcH5b and Hsc70. These data suggest a model by which phosphorylated tau is bound to a chaperone and the complex is recognized by the E3 Ub ligase CHIP.

CHIP can also ubiquitinate phosphorylated tau cultured cells. EGFP-tau, myc-CHIP, FLAG-Ub, and GSK-3beta, or a dominant negative kinase dead GSK-3beta with the K85R mutation [109], were transfected into COS7 cells. Before harvesting, the cells were treated with the proteasome inhibitor MG132 or dimethylsulfoxide (DMSO) as a control for 6 h. Cell lysates were immunoprecipitated with anti-FLAG or polyclonal anti-myc and immunoblotted with monoclonal anti-myc, anti-GSK-3beta or anti-Tau (5E2 and AT8) antibodies. We observed high molecular weight polyubiquitinated tau stained with 5E2 and AT8 but not with Tau1 in immunoprecipitates with anti-FLAG or anti-myc antibodies when EGFP-tau, GSK-3beta, and myc-CHIP were expressed, but not in the absence of myc-CHIP, GSK-3beta, or EGFP-tau. Based on the antibody specificities, this finding suggested that residues S199, S202 and T205 of high molecular weight tau were phosphorylated and contribute to the recognition by the Hsc70/CHIP complex. The structural difference among the tau phospho-isoforms is unknown; however, these data suggest that certain tau phosphorylation events trigger Hsc70 binding. Dou et al. [110] reported that Hsp70 and Hsp90 bind tau, however, they did not examine the phosphorylation state of tau. Presumably, the tau-Hsc70 complex is recognized by CHIP. MG132 treatment enhanced accumulation of polyubiquitinated phosphorylated tau. Loss of function CHIP with an H260Q mutation in the U box and other ubiquitin ligases, such as parkin and cbl, were unable to ubiquitinate tau. Thus, CHIP specifically serves as the E3 ligase for phosphorylated tau.

One group has reported that Parkin can serve as an E3 ligase for tau [111]. Our findings run counter to this result for the following reasons: Tau is not present in co-immunoprecipitates from normal control or Alzheimer brain homogenates with two different anti-Parkin antibodies. These same conditions did co-immunoprecipitate synuclein [90]. Furthermore, purified soluble tau and insoluble tau from Alzheimer brain and normal control brain did not bind myc-Parkin in a pull-down assay. Similarly, Parkin is not present in the cofactor fraction used for in vitro ubiquitination.

Given the many similarities between the tauopathies and synucleinopathies, it is notable that CHIP is present in a complex consisting of Parkin, its substrate Pael, and Hsp70 [112]. However, as of this writing CHIP has not been directly linked to synuclein. CHIP promoted the dissociation of Hsp70 from Parkin and Pael-R, thus facilitating Parkin-mediated Pael-R ubiquitination. Imai et al. claimed that CHIP enhanced Parkin-mediated in vitro ubiquitination of Pael-R even in the absence of Hsp70.

6. The Hsc70/CHIP complex reduces the toxicity of phosphorylated tau

Co-expression of EGFP-tau and GSK-3β caused cell death in COS7 compared to either one of these constructs alone. However, when myc-CHIP and FLAG-Ub were included in the transfection, the amount of cell death decreased significantly. Immunoblots of these transfected cells showed that FLAG-ubiquitinated phosphorylated tau, labeled with 5E2 and AT8 but not Tau1, was most

abundant in cells that had been rescued by myc-CHIP. In contrast, phosphorylated non-ubiquitinated tau was most abundant in cells, which expressed EGFP-tau, GSK-3β and FLAG-Ub, but not myc-CHIP. These results suggested that accumulation of soluble phosphorylated tau is toxic, while insoluble ubiquitinated phosphorylated tau is less toxic. CHIP appears to ubiquitinate phosphorylated tau not only for degradation in the proteasome, but also to move tau into a segregated insoluble fraction, possibly for prevention of cell death. Pretreatment of cells with MG132 before lysis resulted in the accumulation of polyubiquitinated phosphorylated tau in the 1% Triton-X insoluble fraction. Consistent with the protective role for the partitioning of polyubiquitinated phosphorylated tau into an insoluble compartment was the very minimal increase in the death of cells treated with MG132 after transfection with EGFP-tau, GSK-3β and myc-CHIP. The data raise the possibility that insoluble ubiquitinated tau may not directly contribute to cell death.

Interestingly, filamentous green fluorescent bundles were observed in the cytoplasm of COS7 cells transfected with EGFP-tau, FLAG-Ub, myc-CHIP, and mutant GSK-3β. Single transfections with EGFP-tau showed co-localization to the microtubules. In contrast, fluorescence micrographs of EGFP-tau, FLAG-Ub, myc-CHIP, and wild-type GSK-3β transfected cells treated with MG132 revealed green fluorescent aggregates in the cytoplasm. Transfections of these additional cDNAs in various combinations indicated that myc-CHIP was required for the formation of the aggregates. Ubiquitination may transform toxic phosphorylated tau protofibrils or oligomers into large more neutral cellular inclusions.

Under normal conditions, CHIP cooperates with BAG-1, a ubiquitin domain protein that couples Hsc/Hsp70 and the proteasome [113]. BAG-1 directly interacts with CHIP and accepts substrates from Hsc/Hsp70 for presentation of associated proteins to the CHIP ubiquitin conjugation machinery [107]. Therefore, BAG-1 promotes CHIP-induced degradation as shown for the glucocorticoid hormone receptor in vivo. Presumably, if the Hsc/Hsp70 chaperone system fails to restore correct protein folding, the ubiquitin domain protein BAG-1 and the CHIP ubiquitin ligase can shift the activity of the Hsc/Hsp70 chaperone system from protein folding to degradation.

7. Association of PHF tau with Hsp27

With the methodology described above in which human brain homogenates were passed over an affinity column with nickel-beads coupled to recombinant four repeat His-tau phosphorylated by GSK-3β, we eluted a 27-kDa band. This band was identified by mass spectrometry to contain a sequence which exactly matched the sequence of Hsp27 and was immunoreactive to an Hsp27 antibody [90]. For the specificity of the interaction we used purified recombinant Hsp27 and Hsp27 that was MAPKAP kinase 2-phosphorylated (pHsp27). Recombinant His-tau that was either GSK-3β phosphorylated or a control that was non-phosphorylated was incubated with human brain homogenates and the precipitated proteins were immunoblotted with antibodies against Hsp27 and pHsp27. Considerably larger amounts of Hsp27 and pHsp27 were precipitated by phosphorylated tau compared to dephosphorylated tau. This result confirmed that Hsp27 binds directly to phosphorylated tau.

Hsp27 also bound to AD type pathological hyperphosphorylated tau known as PHF tau [90]. PHF tau was purified from AD brain and a fraction of the sample was dephosphorylated by PP2A for use as a negative control. The amount of Hsp27 and pHsp27 in the precipitates of PHF tau was higher than that of dephosphorylated PHF tau, indicating that phosphorylation of PHF tau was a recognition signal for both Hsp27 and pHsp27. The association of Hsp27 and phosphorylated tau was confirmed by co-immunoprecipitation experiments. Hsp27 co-precipitated with tau antibody from homogenates of AD brain, but not from normal brain. The reverse co-immunoprecipitation demonstrated that pathological hyperphosphorylated tau co-precipitated with Hsp27 from the homogenates of AD brain, but not normal brain. These co-immunoprecipitations and reverse co-immunoprecipitations strongly suggested that Hsp27 preferentially bound to pathological hyperphosphorylated tau in human brain tissue.

8. A protective effect of the Hsp27/phosphorylated tau complex

To study the role of this complex in cells, we utilized the BioPORTER Reagent, which allowed us to introduce large complexes into living cells. Soluble PHF tau from AD brain was delivered into HCN2A cells [90]. Based on minimal reaction product when blotted with AT8 24 h later, the cells contained only a small amount of pathological hyperphosphorylated tau. This finding suggested that the activity of phosphatases in the HCN2A cells removed the phosphates from the soluble PHF tau that was bioportaged into the cells. Therefore, the cells were incubated with calyculin-A and okadaic acid, inhibitors of PP1 and PP2A, to inhibit dephosphorylation of pathologically phosphorylated tau after delivery to the intracellular compartment. Consistent with the dephosphorylation of the PHF-tau, TRITC-PHF tau distributed in a typical microtubule pattern, while calyculin A and okadaic acid were distributed more diffusely. Interestingly, when Hsp27 and pHsp27 were delivered with the PHF tau, the amount of pathological hyperphosphorylated tau was reduced and dephosphorylated tau increased. In the presence of Hsp27 and pHsp27 antibody, also delivered by the BioPorter, the amount of hyperphosphorylated tau increased. This finding suggested that Hsp27 and pHsp27 facilitate the degradation and/or dephosphorylation of pathological hyperphosphorylated tau.

Delivery of PHF hyperphosphorylated tau enhanced cell death. When PHF was introduced with Hsp27 cell death decreased as did the amount hyperphosphorylated tau. This ameliorative effect was eliminated with the introduction of an Hsp27 antibody. Thus, pathological hyperphosphorylated tau can cause cell death and Hsp27 attenuated its toxicity. Several studies have shown that hyperphosphorylated tau can cause apoptosis, and apoptotic cell death has been observed in postmortem AD brain and AD animal models [114,115]. In our experiments, we confirmed that pathological hyperphosphorylated tau caused apoptosis as indicated by activation of an effector caspase (caspase-3). This effect too was mitigated by the delivery of Hsp27. The mechanism by which Hsp27 prevents apoptosis may be caused not only by the interaction between Hsp27 and pathological hyperphosphorylated tau, but also by Hsp27-mediated inhibition of pro-caspase-9 and caspase-3 [116].

David et al. [117] showed that unfolded native tau protein is degraded by the proteasome, but they did not test pathological hyperphosphorylated tau. Our studies showed that Hsp27 facilitated the degradation of pathological hyperphosphorylated tau, which is otherwise not efficiently degraded by the proteasome in the absence of Hsp27. Interestingly, Hsp27 facilitated the degradation of hyperphosphorylated tau without ubiquitination. Notably, treatment with the proteasome inhibitor, lactacystin, did not induce the accumulation of ubiquitinated ptau in the presence of Hsp27. Ubiquitin-independent degradation of hyperphosphorylated tau may be another of the many parallels with α-synuclein [118]. Both proteins are natively unfolded and therefore could bypass the need for ubiquitination and unfolding to enter the 20S proteasome. Denatured, non-ubiquitinated proteins can be degraded by the proteasome [119]. Other proteins such as $p21^{WAF1/CIP1}$ [120] and ornithine decarboxylase [121] can be degraded in a ubiquitin-independent manner.

The cytoprotective effects of Hsp27 include its role as a molecular chaperone, the inhibition of caspase activation, the prevention of stress-induced disruption of the cytoskeleton, and the modulation of the intracellular redox potential [116]. The induction and expression of Hsp27 have been widely studied in the mammalian central nervous system [122]. HSP27 is up-regulated in a transgenic ALS model with an SOD mutation [123]. Hsp27 is highly inducible in cortical astrocytes after seizure activity [124] and ischemic injury [125]. Hsp27 is also normally present in many neurons of the brainstem and spinal cord of rat and mouse [126]. Hsp27 is induced and expressed at very high levels for extended periods of time in vagal trans-section [127]. These results suggest that Hsp27 plays a critical role in neuronal metabolism and neuronal survival. Hsp27 also prevents neuronal cell death in simulated ischemia [128], peripheral nerve trans-section [129,130], and polyglutamine repeat expansion of huntingtin [131]. Phosphorylated tau may have at least two mechanisms leading to its degradation—a ubiquitin-independent pathway that can be facilitated by Hsp27 and a ubiquitin-dependent pathway mediated by the CHIP–Hsc70 complex (Fig. 1) [90].

9. Chaperones and the late-onset foldopathies: a broad perspective

Over the past decade, a common theme has emerged: protein folding abnormalities are a fundamental part of the pathogenetic cascade leading to the phenotype of many

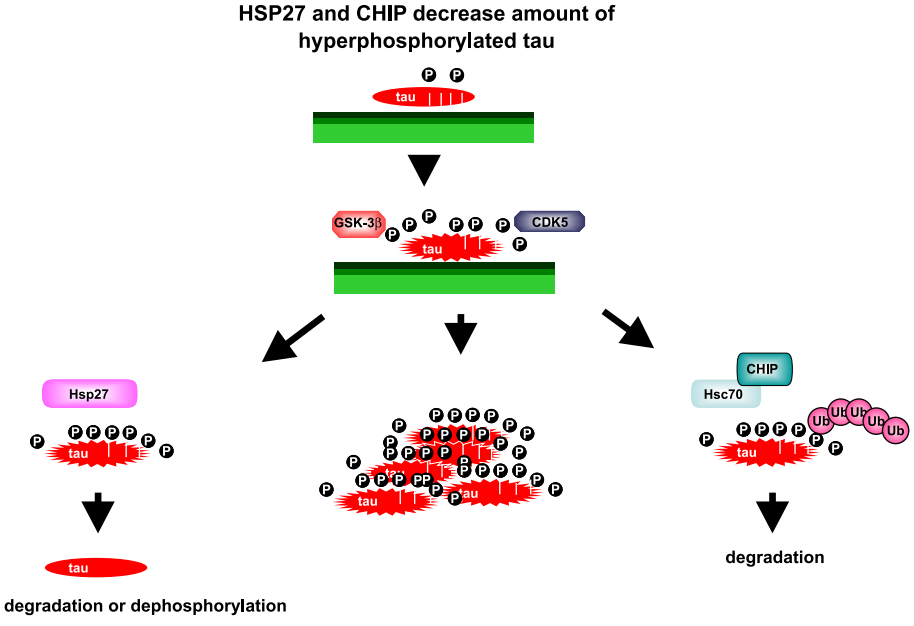

Fig. 1. Phosphorylated tau may have at least two mechanisms leading to its degradation—a ubiquitin-independent pathway that can be facilitated by Hsp27 and a ubiquitin-dependent pathway mediated by the CHIP–Hsc70 complex.

neurodegenerative conditions. So pervasive is this theme that to the classical major disease categories the addition of protein folding disorders or foldopathies is now timely. These diseases inhabit a zone between genetic predisposition, which usually accelerates the phenotype, and sporadic [132]. However, the term sporadic conceals a more subtle polygenic or possibly an epigenetic component to phenotypic expression. Among the genetic forms of neurodegeneration, some autosomal dominant mutations increase the expression levels of the wild-type protein. For example, a triplication upstream of α-synuclein increases its expression and the error results in Parkinson's disease [133,134]. Splice site mutations in the tau gene increase the proportion of the four repeat isoform of tau and this error results in tauopathy. In both cases, the expression of the wild-type protein increases. Therefore, one disease mechanism is related to gene dose, which accelerates disease onset by several decades relative to the sporadic cases. One might assume that the expression levels of tau and alpha-synuclein are regulated within a rather narrow window and excess protein may overwhelm the folding and degradative systems. Whether energy or substrate components within these systems are limiting is unknown. However, it is conceivable that smaller expression increases of these genes could trigger disease onset within the age range that we usually call sporadic. Increased expression may not only be due to polymorphisms in the disease gene, i.e., tau or alpha-synuclein, but to a variety of signaling systems, which regulate their expression levels or the proportion of phosphorylated protein. These set points may not only be due to genetic polymorphisms, but also to epigenetic and environmental factors which contribute to setting kinase activation levels.

One of the perplexing features among the tauopathies is the variety of phenotypes observed secondary to mutations in the tau gene. The tau gene can harbor mutations that lead to quite diverse clinical phenotypes collectively grouped under the designation frontotemporal dementia with parkinsonism linked to chromosome 17 [38]. Queitsch et al. [135] and Rutherford and Lindquist [136] observed that a reduced dosage of HSP90 resulted in the emergence of a surprising variety of phenotypes among genetically heterogeneous stocks. From this observation they proposed that HSP90 could conceal the phenotypes associated with genetic substitutions. The damage induced by a misfolded protein is abrogated by its binding to chaperones and directed toward the proteasome. If this system fails and the aggregate remains segregated within the cell as an insoluble inclusion, damage can be temporarily stayed. Hsp70 expression preserves dopaminergic cells in a *D. melanogaster* model of Parkinson's disease [28] and prevents cellular damage due to the expression of polyglutamine mutants [7,9,137]. However, one possible cost of this strategy is that the aggregate can serve as a sink for chaperone proteins and reduce their cellular levels. Furthermore, HSP induction is reduced in senescence (reviewed in Ref. [45]). In this way, a variety of phenotypes may emerge that depend on previously concealed individual differences.

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