THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 6, Issue of February 6, pp. 4869–4876, 2004
Printed in U.S.A.

CHIP-Hsc70 Complex Ubiquitinates Phosphorylated Tau and Enhances Cell Survival*

Received for publication, June 3, 2003, and in revised form, November 6, 2003
Published, JBC Papers in Press, November 10, 2003, DOI 10.1074/jbc.M305838200

Hideki Shimura‡, Daniel Schwartz§, Steven P. Gygi§, and Kenneth S. Kosik‡¶

From the ‡Department of Neurology, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115 and the §Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

The microtubule-binding protein tau has been implicated in the neurofibrillary pathology of Alzheimer's disease. Within affected cells, ubiquitinated and hyperphosphorylated tau assembles into massive filamentous polymers. Eventually these tangle-bearing neurons die. The formation of neurofibrillary tangles closely parallels the progression and anatomic distribution of neuronal loss in Alzheimer's disease, suggesting that these lesions play a role in the disease pathogenesis. Mutations in the human tau gene cause autosomal dominant neurodegenerative disorders. These and other neurodegenerative conditions are also characterized by extensive neurofibrillary pathology. The mechanisms underlying tau-mediated neurotoxicity remain unclear; however, phosphorylated tau is a strong candidate for a toxic molecule, particularly those isoforms phosphorylated by the kinases glycogen synthase kinase 3β and Cdk5. Here we show that Alzheimer tau binds to Hsc70, and its phosphorylation is a recognition requirement for the addition of ubiquitin (Ub) by the E3 Ub ligase CHIP (carboxyl terminus of the Hsc70-interacting protein) and the E2 conjugating enzyme UbcH5B. Other E3 Ub ligases including parkin and Cbl failed to ubiquitinate phosphorylated tau. CHIP could rescue phosphorylated tau-induced cell death, and therefore the CHIP-Hsc70 complex may provide a new therapeutic target for the tauopathies.

Tau inclusions are a major feature of several neurodegenerative diseases including Alzheimer's disease (1, 2). Normally, tau functions as a neuronal microtubule-binding protein, and its binding to microtubules is regulated both by alternative splicing within the binding region and by phosphorylation in and around the binding region (3). The flanking region around the microtubule-binding repeats is phosphorylated by multiple different kinases. Up to 19 different sites can be phosphorylated on tau (4, 5), thus creating a staggeringly high number of possible tau phosphoisoforms. 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 the controls 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 3β (GSK-3β),[1] extracellular signal regulated kinase, stress-activated protein kinase, cyclin-dependent kinase 5 (Cdk5), CDC2-cyclin A kinase, MARK kinase, Ca^{2+}/calmodulin-dependent protein kinase, cyclic AMP-dependent protein kinase, protein kinase C, casein kinase I and II, double-stranded DNA-dependent protein kinase, microtubule-associated protein/microtubule affinity-regulating kinase, and tau-tubulin kinase (4, 5).

Because phosphorylation releases tau from microtubules and because tau in the paired helical filament (PHF) is highly phosphorylated, kinases have been viewed with interest for a possible role in pathogenesis. Fath *et al.* (6) 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. *In vivo* evidence for an interaction with tau exists for Cdk-5 and GSK-3β. Overexpression of human p25 (an activator of Cdk5) in mice induced tau hyperphosphorylation and cytoskeletal disruptions reminiscent of AD, but no filamentous deposits (7). Noble *et al.* (8) 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 neurofibrillary tangles accompanied these changes as well as an association of active GSK with insoluble tau (8). Overexpression of GSK-3β under the control of a tetracycline-sensitive transactivator also induced tau hyperphosphorylation, somatodendritic mislocalization of tau, and neuronal apoptosis (9). However, the complexity of the role of GSK-3β was underscored in transgenic mice that expressed a constitutively active mutant form of human GSK-3β 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 (10). Nevertheless, overall these studies implicate tau hyperphosphorylation in tau-related neurodegeneration.

Ubiquitination is a cellular process by which short lived or damaged proteins are conjugated with multimers of Ub, mark-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Harvard Institute of Medicine, Brigham and Women's Hospital, 77 Ave. Louis Pasteur, Boston, MA 02115. Tel.: 617-525-5230; Fax: 617-525-5252; E-mail: kkosik@rics.bwh.harvard.edu.

[1] The abbreviations used are: GSK, glycogen synthase kinase; PHF, paired helical filament; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; MES, 4-morpholineethanesulfonic acid; GST, glutathione *S*-transferase; PP2A, protein phosphatase 2A; EGFP, enhanced green fluorescence protein; AD, Alzheimer's disease.

ing 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. Evidence in favor of the Ub-proteasome system involvement in the pathogenesis of neurodegeneration includes the following: 1) inclusion bodies found in a variety of neurodegenerative disorders contain Ub and ubiquitinated proteins; 2) mutations in two Ub pathway enzymes, parkin (11–14) and ubiquitin carboxyl-terminal hydrolase L1 (15, 16), cause a form of Parkinson's disease (PD); 3) proteasome activity is decreased or impaired in PD and AD post-mortem brain (17); 4) a mutation in a multi-Ub gene is associated with AD (18); and 5) the loss of function of the Ub ligase, Ube3a in SCA1 mice increases neurodegeneration (19).

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 (20). We therefore hypothesized that hyperphosphorylated tau could be ubiquitinated by an unknown E3 Ub ligase(s) for proteasomal delivery and degradation. Here we show that a CHIP (carboxyl terminus of the Hsc70-interacting protein)-Hsc70 complex selectively ubiquitinates phosphorylated tau in collaboration with UbcH5B. Furthermore, CHIP attenuates phosphorylated tau-induced cell death.

MATERIALS AND METHODS

Cells and Transfection—COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 20 mM HEPES, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum in a 5% CO_2 environment. Cells were transfected with various expression vectors using the LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

Immunopurification, Immunoblotting, and Immunocytochemistry—Temporal cortex specimens (2 g) from human brain were homogenized using the lysis buffer and sedimented at 20,000 $\times$ *g* for 1 h at 4 °C. Supernatants were incubated with protein A and protein G-Sepharose (Pierce) cross-linked to tau antibodies for 2 h at 4 °C. For immunoblotting, transfected cells were lysed in the lysis buffer, and the extracts were centrifuged at 20,000 $\times$ *g* for 1 h at 4 °C. The supernatants were referred to as the 1% Triton X-100-soluble fraction. The pellets were further resolved in lysis buffer containing 4% SDS and centrifuged at 20,000 $\times$ *g* for 1 h at 22 °C. The supernatants were referred to as the 1% Triton X-100-insoluble fraction. 4$\times$ SDS in sample buffer was added to the lysates, and immunoprecipitation, PAGE, and immunoblots were performed as described (21). We washed immunoprecipitated beads by 1 M NaCl, and the fraction that was released from beads was named as the co-factor fraction. For immunocytochemistry, the cells were washed with phosphate-buffered saline, fixed with 4% formaldehyde, and then stained with anti-FLAG (Sigma) antibody or AT8 (Pierce). Primary antibody was localized by secondary antibody conjugated to Texas Red (Jackson ImmunoResearch Laboratory, West Grove, PA) at room temperature for 1 h. Live and dead cells were distinguished with the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions. Stained cells were analyzed with a laser-scanning confocal microscope system (Zeiss, Heidelberg, Germany).

In Vitro Tau Phosphorylation, Dephosphorylation, and Deglycosylation Assay—Active recombinant GSK-3β was incubated in 30 μl of reaction mixture containing 10 μM ATP, recombinant His-tau, and kinase buffer. Immunoprecipitated tau was incubated with *N*-glycosidase or *O*-glycosidase as per the manufacturer's protocol (Pro Zyme, San Leandro, CA). Immunoprecipitated tau was incubated with protein-tyrosine phosphatase 1B or protein phosphatase 2A (PP2A) for 6 h at 37 °C.

In Vitro Ubiquitination Assay of Immunoprecipitated Tau—We eluted immunoprecipitated tau from antibodies that were chemically conjugated to protein A- and G-Sepharose beads, and proteins were eluted from the beads with 0.2 M glycine, pH 2.8, and the beads were removed. Then pH (acidic to neutral) was collected by 0.1 M Tris-HCl, pH 8.5. Immunoprecipitated tau was incubated in a 100-μl reaction mixture containing 200 ng of Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) fraction, or 4 μg of each E2, 2 μg of FLAG-Ub, and reaction buffer (50 mM Tris-HCl, pH 7.5, 4 mM ATP, and 2 mM $MgCl_2$) for 30 min at 37 °C. To release the co-factor fraction from immunoprecipitated tau, we washed the beads with 1 M NaCl before performing the *in vitro* ubiquitination assay. After the *in vitro* ubiquitination assay, FLAG-ubiquitinated proteins were immunoprecipitated with anti-FLAG antibody. The precipitates were analyzed by immunoblotting with various antibodies.

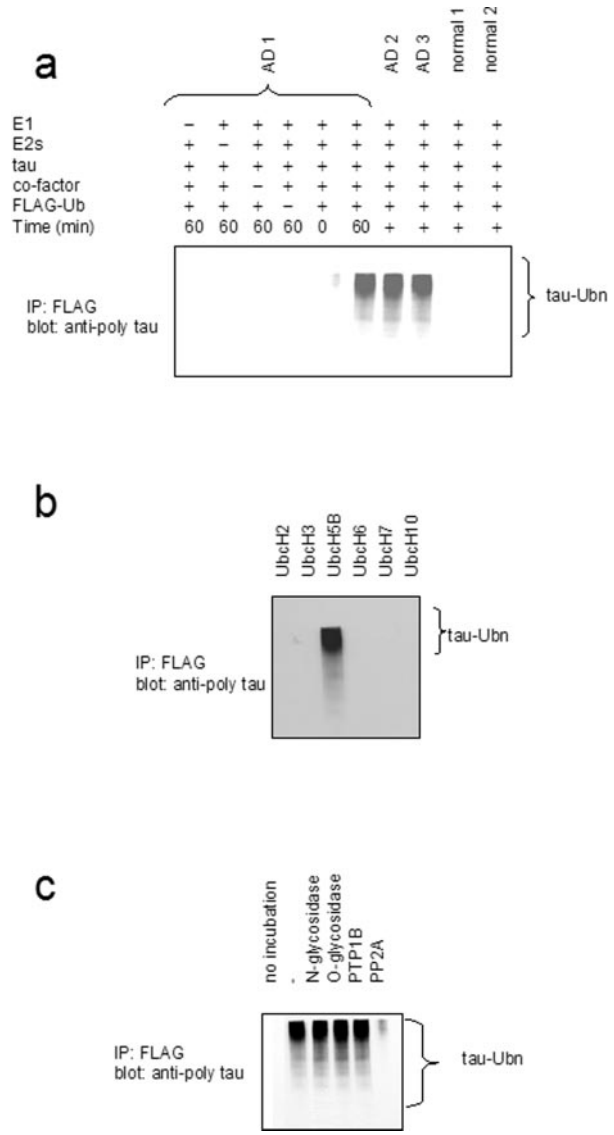

FIG. 1. **Ubiquitination of tau from AD brain *in vitro*.** *a*, brain homogenates of temporal cortex from three AD and two normal cases were immunoprecipitated with a mixture of anti-tau antibodies 5E2 and the polyclonal tau antibody. Immunoprecipitated tau from AD or normal brain homogenates were incubated with the indicated combinations of FLAG-Ub, E1, and E2s and subsequently immunoprecipitated with a FLAG antibody. The co-factor refers to immunoprecipitated beads that were washed with 1 M NaCl before *in vitro* ubiquitination. High M_r tau immunoreactivity (*tau-Ubn*) was detected with the polyclonal tau antibody. *b*, immunoprecipitated tau from AD brain homogenate was incubated with E1, FLAG-Ub, and various E2s. *c*, immunoprecipitated tau from AD brain was incubated with *N*-glycosidase, *O*-glycosidase plus sialidase A, protein-tyrosine phosphatase (*PTP1B*), or PP2A. PP2A treatment inhibited ubiquitination of immunoprecipitated tau from AD brain.

In Vitro Ubiquitination Assay of Recombinant His-Tau—His-tau and phosphorylated His-tau by GSK-3β was incubated in a 100-μl reaction mixture containing 200 ng of E1, 4 μg of UbcH5B, 2 μg of FLAG-Ub, co-factor fraction or 2 μg of GST-CHIP and 1 μg of Hsc70, and reaction buffer (50 mM Tris-HCl, pH 7.5, 4 mM ATP, and 2 mM $MgCl_2$) for 30 min at 37 °C.

Purification of Ub Ligase for Phosphorylated Tau—Temporal cortex specimens (100 g) from normal human brain were homogenized using lysis buffer (50 mM MES, 0.5 mM $MgCl_2$, and 2 mM EGTA, pH 6.5, containing 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM sodium

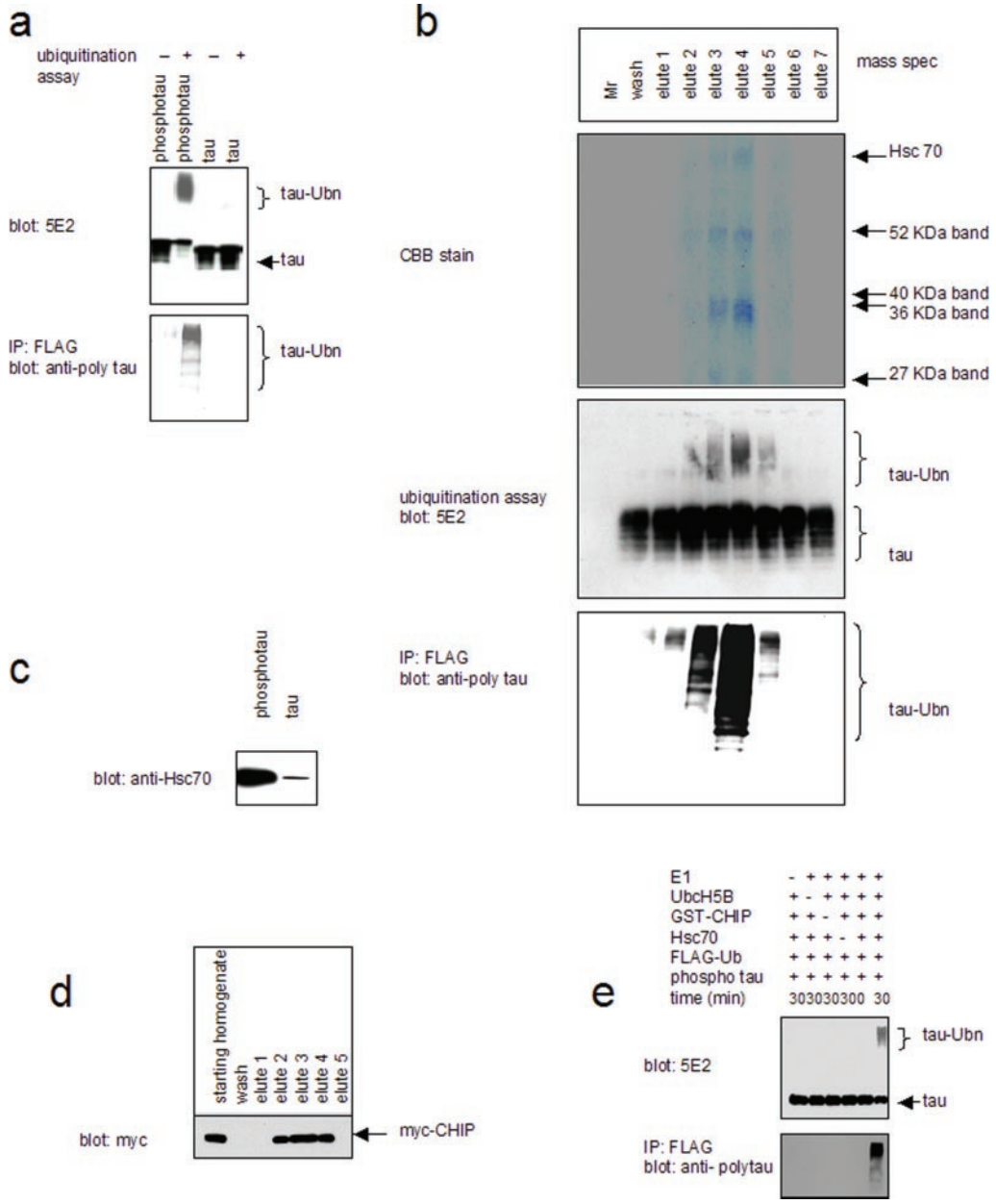

FIG. 2. **CHIP serves as a ubiquitin ligase for phosphorylated tau *in vitro*.** *a*, recombinant His-tau was phosphorylated by GSK-3β, and *in vitro* ubiquitination was performed using FLAG-Ub, E1, UbcH5B, and the co-factor fraction from AD tau immunoprecipitates. *b*, normal brain homogenate was passed through the two affinity columns: a phosphorylated His-tau column and GST-UbcH5B column. The eluate was separated by SDS-PAGE and stained with Coomassie Brilliant Blue. *In vitro* phosphorylated tau is ubiquitinated by the eluate. Phosphorylated tau was incubated with FLAG-Ub, E1, and UbcH5B and then immunoprecipitated with a FLAG antibody. High M_r tau immunoreactivity was detected by the polyclonal tau antibody. *c*, His-tau or His-phosphorylated tau was incubated with human brain homogenates and washed. The pull-down was probed with an Hsc70 antibody. *d*, COS7 cells were transfected with a Myc-CHIP expression vector, and the lysate was passed through the two affinity columns. The elution was immunoblotted with anti-Myc antibody. *e*, *in vitro* phosphorylated tau is ubiquitinated by recombinant GST-CHIP. Phosphorylated tau was incubated with FLAG-Ub and the indicated combination of E1, UbcH5B, GST-CHIP, and Hsc70 and immunoprecipitated with a FLAG antibody. High M_r tau immunoreactivity was detected by 5E2.

pyrophosphate, and 20 mM NaF) in the presence of EDTA-free protease inhibitors (Roche Applied Science) and sedimented at 20,000 $\times g$ for 1 h at 4 °C. The supernatants were passed over a phosphorylated His-tau (200-μg) column first, and then a GST-UbcH5B (100-μg) column followed by a wash in PBS and then fractionated and eluted with 1 M NaCl. Eluted proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Coomassie Brilliant Blue-stained gels were sliced and analyzed by mass spectrometry. Ubiquitin ligase activity of the elutions was analyzed by an *in vitro* phosphorylated tau ubiquitination assay.

His-Tau Pull-down Assay—His-tau or phosphorylated His-tau (2 μg) coupled to nickel beads was added to normal human brain homogenates and incubated at 4 °C for 1 h with shaking. The proteins were eluted from the beads by adding 40 μl of 2× Laemmli buffer.

Antibodies—The antibodies used were 5E2 (22, 23) tau antibody, which recognizes all isoforms of tau; AT8 (24) (Pierce), a tau antibody that recognizes phosphorylated residues Ser^{202} and Thr^{205}; PHF1 (25, 26), a tau antibody that recognizes phosphorylated residues Ser^{396} and Ser^{404}; Tau 1 (24), a tau antibody that recognizes residues Ser^{199}, Ser^{202}, and Thr^{205} when nonphosphorylated; a polyclonal tau antibody (Sigma) that recognizes all isoforms of tau; anti-FLAG antibody (Sigma); anti-Hsc70 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-Myc antibody (Santa Cruz Biotechnology); anti-Cbl antibody (Santa Cruz Biotechnology); and GSK-3β antibody (Zymed Laboratories Inc., South San Francisco, CA).

Plasmids and Recombinant Proteins—FLAG-Ub and Myc-parkin vectors were generated as described previously (12). EGFP-tau was generated as described previously (27). HA-Cbl, Myc-CHIP, mutant Myc-CHIP, and recombinant GST-CHIP were generous gifts from Dr. Band. HA-GSK3β was a gift from Dr. Woodgett. GSK-3β was cloned

into pcDNA 3.1(+) Vector. Mutant GSK-3β K85R was cloned into pAdTrack vector using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). Recombinant His-tau, FLAG-Ub, E1, E2s, GSK-3β, Hsc70, PP2A, and protein-tyrosine phosphatase 1B were purchased from Sigma.

RESULTS

AD Brain Tau Is Polyubiquitinylated in Collaboration with UbcH5B—To determine whether tau from human brain is ubiquitinated *in vitro*, we carried out co-immunoprecipitation experiments on homogenates of frozen frontal cortex from two normal brains and three AD brains. We used a tau antibody mixture that included 5E2 (22, 23) and affinity-purified polyclonal tau antibodies. Tau was extracted using 1% Triton X-100, and therefore the more insoluble forms of tau-associated PHFs would not be solubilized. As observed by others, both three- and four-repeat tau proteins were immunoprecipitated; however, proportionally considerably more four-repeat tau was present. We eluted tau from the tau antibodies and carried out an *in vitro* ubiquitination reaction, which utilized commercially available mammalian E1, E2 fractions, and FLAG-Ub. We immunoprecipitated FLAG-Ub-conjugated molecules with anti-FLAG antibody beads and probed with the polyclonal tau antibody. Interestingly, in an *in vitro* ubiquitination reaction, AD tau was ubiquitinated, and control tau was not (Fig. 1*a*).

This procedure utilized a co-factor fraction obtained by washing the immunoprecipitated tau in 1 M NaCl. A fraction obtained by washing the immunoprecipitated beads in 1 M NaCl before tau was eluted did not ubiquitinate tau *in vitro* (Fig. 1*a*). Furthermore, immunoprecipitated tau before undergoing *in vitro* ubiquitination, was not ubiquitinated. This experiment indicated that the co-factor fraction contained an E3 Ub ligase for tau. The high molecular weight band in these experiments was ubiquitinated tau, and not aggregated tau, because the experiment, when done without FLAG-Ub, did not reveal a high molecular weight band with the polyclonal tau antibody.

The *in vitro* ubiquitination of AD tau, but not control tau, was based on reactions that utilized commercially available E1 and E2 fractions. We substituted single specific E2s in the *in vitro* ubiquitination reaction and found that AD tau was intensely and consistently ubiquitinated with UbcH5B (Fig. 1*b*). The other E2s tried in the reaction mixture did not ubiquitinate AD tau. This result strongly suggested that the E2 conjugating enzyme for tau ubiquitination is UbcH5B. High molecular weight tau polyubiquitination products obtained by using UbcH5B in the *in vitro* ubiquitination reactions were observed only with tau that was immunoprecipitated from AD and not from normal control brain, as expected (data not shown).

Phosphorylation of Tau Is the Signal for Tau Ubiquitination—The observations above led to the question of what modifications of tau are recognized by the E3 Ub ligase. The most likely modifications are phosphorylation and/or glycosylation (4, 5). Our experiments showed that AD type phosphorylation of tau is the signal for tau ubiquitination. AD tau was immunoprecipitated from brain and treated with PP2A, protein-tyrosine phosphatase 1B, *N*-glycosidase, sialidase, or *O*-glycosidase and then *in vitro* ubiquitinated, immunoprecipitated with anti-FLAG antibodies, and finally incubated with the polyclonal tau antibody. High molecular weight polyubiquitinated tau can be seen in all lanes except in the control (no incubation) and in the PP2A-treated lane (Fig. 1*c*). These data indicated that phosphorylation of PP2A dephosphorylation sites is an important recognition signal for ubiquitination. This finding lends further support to the importance of tau phosphorylation in tangle formation whether in AD or in the tauopathies.

Alzheimer Tau Is Bound to Hsc-70—We first focused on the co-factor fraction to identify the tau E3 Ub ligase. Initially,

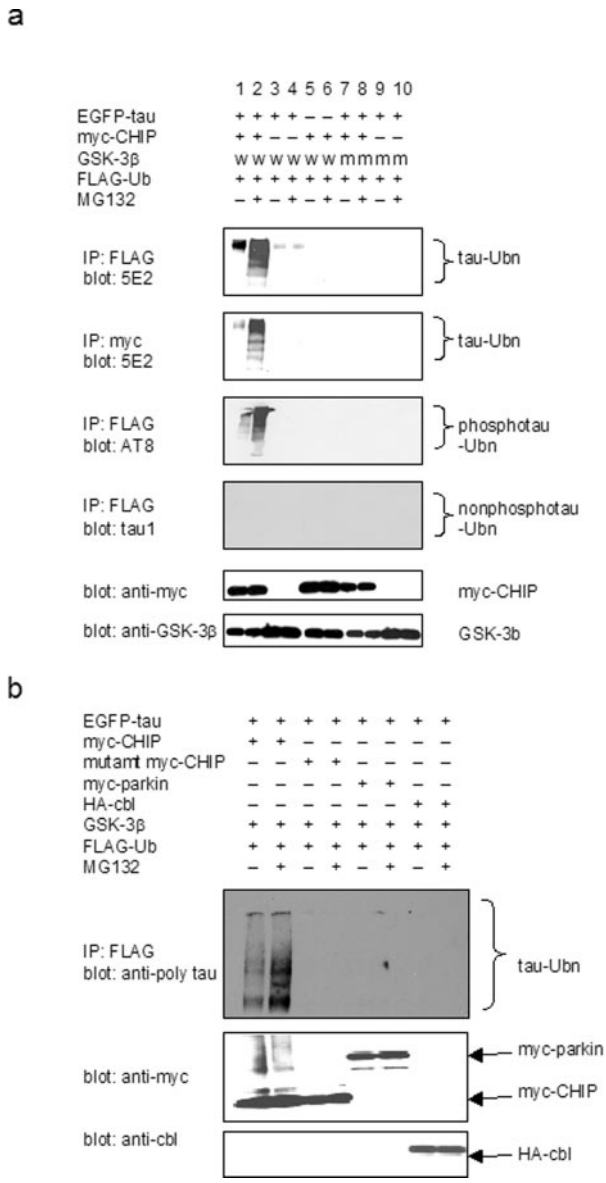

FIG. 3. **Overexpression of CHIP induces phosphorylated tau ubiquitination *in vivo*.** *a*, COS7 cells were transfected with a plasmid encoding FLAG-Ub and the indicated combination of plasmids encoding EGFP-tau, Myc-CHIP, and mutant (*m*) or wild-type (*w*) GSK-3β. 48 h after transfection, the cells were treated with 50 μM MG132 or Me_2SO as a control for 6 h before cell lysis. Transfected cell lysates and anti-FLAG or anti-Myc immunoprecipitates were resolved by SDS-PAGE and immunoblotted with the 5E2, AT8, and tau1 antibodies. *b*, COS7 cells were transfected with vector encoding FLAG-Ub and GSK-3β together with Myc-CHIP, mutant Myc-CHIP, Myc-parkin, or HA-Cbl. 48 h after transfection, the cells were treated with 50 μM MG132 or Me_2SO as a control for 6 h before cell lysis. Anti-FLAG immunoprecipitates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

proteins in the co-factor fraction were visualized by gel electrophoresis, and those bands, which were uniquely observed in the AD tau immunoprecipitation, were analyzed by mass spectrometry. However, we could not identify the E3 Ub ligase in this experiment (data not shown). We then refined the strategy based on the finding that PP2A can eliminate recognition by the E3 Ub ligase and prevent *in vitro* ubiquitination. PP2A is very abundant in brain and is associated with microtubules (28). 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 (29). Many of the sites that are dephosphorylated by PP2A are

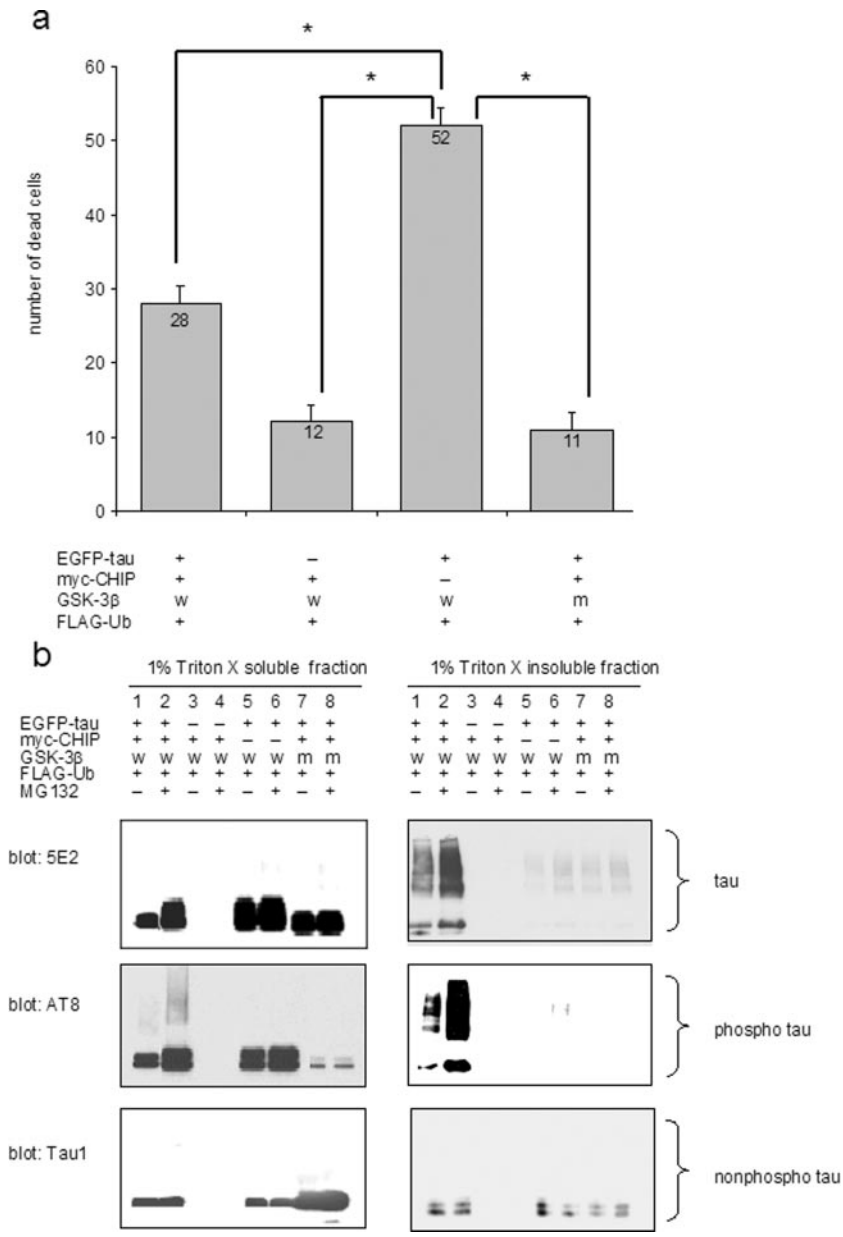

FIG. 4. **Exogenously expressed CHIP rescues cell death caused by phosphorylated tau.** Transfected cells were prepared as in Fig. 3. *a*, data are shown as dead cell counts from six randomly selected microscopic fields in each of three replicate cultures (hence $n = 18$), and statistical significance was assessed using one-way analysis of variance with Student-Neuman-Kuells *post hoc* tests between each group (*, $p < 0.05$). Error bars indicate mean ± S.D. Transfections were with either wild type (*w*) or mutant (*m*) GSK-3β. *b*, transfected cells were lysed with buffer containing 1% Triton-X, and the supernatants and pellets were separated by centrifugation. The supernatant was referred to as 1% Triton X-soluble fraction, and the pellet was referred to as 1% Triton X-insoluble fraction. These fractions were resolved by SDS-PAGE and immunoblotted with the indicated tau antibodies.

phosphorylated by either GSK-3β or Cdk5. These include sites Ser^{199}, Ser^{202}, Thr^{205}, Ser^{396}, and Ser^{404} (30–33). We used 200 μg of amino-terminal His-tagged full-length recombinant human tau in an *in vitro* phosphorylation reaction with GSK-3β. When phosphorylated, the tau protein reacted on immunoblots with PHF1 (25, 26) and AT8 (24), indicating that at least sites Ser^{202}, Thr^{205}, Ser^{396}, and Ser^{404} were phosphorylated. Following GSK-3β incubation, this tau served as an excellent substrate for *in vitro* ubiquitination using UbcH5B and the cofactor fraction from AD tau immunoprecipitates (Fig. 2*a*). This finding suggested that GSK-3β can place phosphates on tau that create recognition sites for an E3 Ub ligase. The finding does not imply that GSK-3β has this property exclusively; in fact, Cdk5 can also place phosphates on tau that serve as an E3 Ub ligase recognition signal, because tau phosphorylated by Cdk5 was also ubiquitinated *in vitro* (data not shown).

Human recombinant N-terminal His-tau was phosphorylated by GSK-3β and bound to a nickel column. Normal human brain extract was passed over the column. The bound fraction was eluted and passed over a column bound to amino-terminal GST-UbcH5B. When the fraction bound to this column was eluted, it retained E3 Ub ligase activity for the recombinant phosphorylated tau substrate (Fig. 2*b*). The sample contained five bands by Coomassie Brilliant Blue staining (from 27 to 70 kDa) (Fig. 2*b*), and each of these bands was analyzed by mass spectroscopy. The analysis revealed that the 70-kDa band was the heat shock cognate 70-kDa protein (Hsc70).

Furthermore, we examined the specificity of the interaction between phosphorylated tau and Hsc70. We conjugated phosphorylated His-tau and nonphosphorylated His-tau to nickel beads and added homogenates of temporal cortex from normal human brain. We found a large amount of Hsc70 in the pull-down with phosphorylated His-tau conjugated to the nickel beads, and considerably more Hsc70 compared with the nonphosphorylated His-tau, suggesting that Hsc70 predominantly binds phosphorylated tau (Fig. 2*c*).

In Vitro Ubiquitination of the CHIP-Hsc70-phosphorylated Tau Complexes—The presence of Hsc70 in the complex suggested a degradation pathway that utilizes CHIP as the E3 ligase (34). Therefore, the fraction eluted from the GST-UbcH5B column was immunoblotted with an antibody against CHIP (Affinity BioReagents, Golden, CO) and an immunoreactive band of 32 kDa was observed (data not shown). However, because this antibody lacked specificity, we examined whether

exogenously expressed Myc-CHIP can be eluted from an affinity-phosphorylated His-tau column and GST-UbcH5B column. We transfected Myc-CHIP into COS7 cells, and lysates from cells were passed over the phosphorylated His-tau and GST-UbcH5B columns and eluted by 1 M NaCl. The elution was analyzed by immunoblotting with a Myc antibody, and we detected Myc-CHIP (Fig. 2*d*). 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. We further examined whether recombinant GST-CHIP ubiquitinates phosphorylated tau *in vitro* in the presence of UbcH5B and Hsc70. Ubiquitination of phosphorylated tau was detected as high molecular weight bands by immunoblotting with 5E2 in the presence of UbcH5B and Hsc70 (Fig. 2*e*).

CHIP Ubiquitinates Phosphorylated Tau for Proteasomal Degradation in Vivo—Next, we tested whether CHIP ubiquitinates phosphorylated tau *in vivo*. We transfected EGFP-tau, Myc-CHIP, FLAG-Ub, and GSK-3β or a kinase-dead GSK-3β with the K85R mutation (35) into COS7 cells. Before harvesting, the cells were treated with the proteasome inhibitor MG132 or Me_2SO as a control for 6 h. Lysates from cells were dissolved in 1% Triton X-containing buffer, immunoprecipitated with anti-FLAG or polyclonal anti-Myc, and immunoblotted with monoclonal anti-Myc, anti-GSK-3β, 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-3β, and Myc-CHIP were expressed, but not in the absence of Myc-CHIP, GSK-3β, or EGFP-tau (Fig. 3*a*). The phosphorylation state of the high molecular ubiquitinated tau was probed; AT8, which recognizes phosphorylated Ser^{202} and Thr^{205}, and 5E2, which recognizes all isoforms of tau in a phosphorylation-independent manner were reactive with high molecular weight ubiquitinated tau. Tau1, which recognizes dephosphorylated Ser^{198}, Ser^{199}, Ser^{202}, and Thr^{205} was not reactive. This finding suggested that residues Ser^{199}, Ser^{202}, and Thr^{205} of high molecular weight tau were phosphorylated, and MG132 treatment enhanced accumulation of polyubiquitinated phosphorylated tau. These results indicate that CHIP specifically ubiquitinates phosphorylated tau for degradation by the proteasome *in vivo*.

We next examined whether loss of function CHIP with an H260Q mutation in the U box or whether other ubiquitin ligases, such as parkin and Cbl, were able to ubiquitinate tau. EGFP-tau, GSK-3β, and FLAG-Ub were co-expressed with Myc-CHIP, mutant Myc-CHIP, Myc-parkin, or HA-Cbl in COS7 cells treated with MG132 or Me_2SO as a control. Cell lysates were probed with a Myc antibody or an HA antibody and immunoprecipitated with a FLAG antibody after immunoblotting with the polyclonal tau antibody (Fig. 3*b*). Prominent high molecular weight tau was observed in anti-FLAG immunoprecipitates of cell lysates from Myc-CHIP, but not mutant Myc-CHIP, Myc-parkin, and HA-Cbl. Thus, CHIP specifically serves as the E3 ligase for phosphorylated tau.

CHIP Rescues the Phosphorylated Tau-induced Cell Death—We also observed that expression of both EGFP-tau and GSK-3β caused cell death. Therefore, we transfected EGFP-tau, GSK-3β, Myc-CHIP, and FLAG-Ub into COS7 cells to determine the effect of CHIP-mediated ubiquitination of tau on cell survival. We distinguished dead from live cells with a LIVE/DEAD viability/cytotoxicity kit (Molecular Probes) and counted dead cells in each field 40 h after transfection. We found that the expression of both EGFP-tau and GSK-3β significantly increased the amount of cell death compared with the expression of EGFP-tau and mutant GSK-3β ($p < 0.005$) or GSK-3β alone ($p < 0.005$) (Fig. 4*a*). This observation indicated

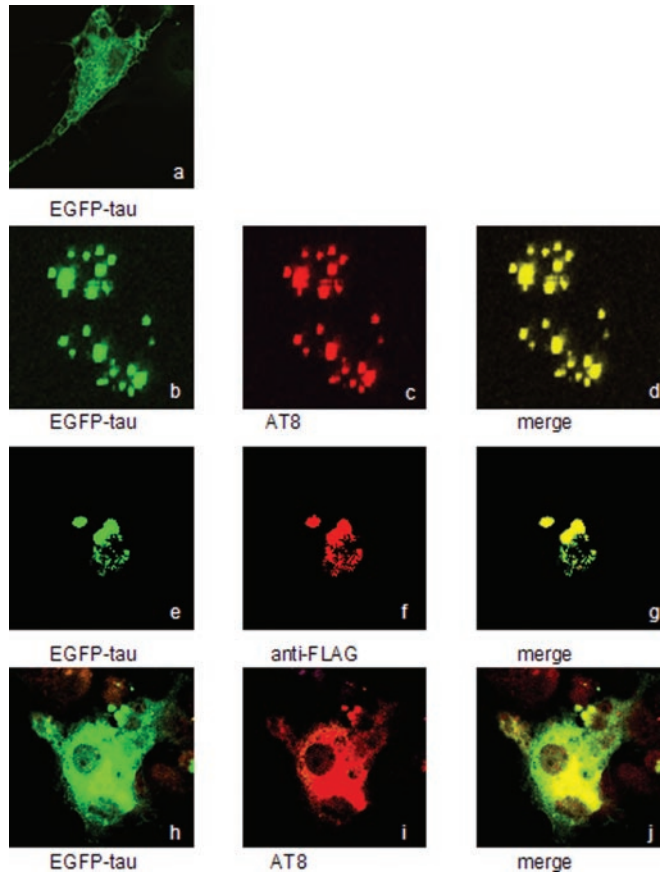

FIG. 5. **EGFP-tau aggregates in transfected cells.** COS7 cells were fixed and stained with AT8 or anti-FLAG antibody. The *green signal* is EGFP-tau (*a*, *b*, *e*, and *h*), and the *red signal* is phosphorylated tau (*c* and *i*) or FLAG ubiquitin (*f*). *a*, cells were transfected with EGFP-tau, mutant GSK-3β, Myc-CHIP, and FLAG-Ub. *b–g*, the cells were transfected with EGFP-tau, GSK-3β, Myc-CHIP, and FLAG-Ub. *h–j*, the cells were transfected with EGFP-tau, GSK-3β, and FLAG-Ub. These cells were treated with 50 μM MG132 for 4 h.

that cell death depended on the expression of GSK-3β and phosphorylated tau, and phosphorylated tau conferred cell toxicity. Interestingly, exogenously expressed Myc-CHIP decreased the amount of cell death caused by the co-expression of EGFP-tau and GSK-3β ($p < 0.005$). This finding suggested that phosphorylated tau may cause cell death and that CHIP can rescue this type of cell death.

The transfected cells described above were analyzed by immunoblots (Fig. 4*b*). Cells were lysed in 1% Triton-X, and the insoluble pellets were further solubilized in 4% SDS-containing buffer. High molecular weight FLAG-ubiquitinated phosphorylated tau, labeled with 5E2 and AT8 but not Tau1, was most abundant in cells that had been rescued by Myc-CHIP. Cells that expressed EGFP-tau, Myc-CHIP, GSK-3β, and FLAG-Ub had reduced cell death (Fig. 4*a*) and increased FLAG-ubiquitinated phosphorylated tau in both the 1% Triton X-insoluble fraction and the anti-FLAG immunoprecipitates. In contrast, phosphorylated tau (not ubiquitinated) was most abundant in the 1% Triton X-soluble fraction of the cells, which expressed EGFP-tau, GSK-3β, and FLAG-Ub but not Myc-CHIP and had a large amount of cell death. These results suggest that accumulation of soluble phosphorylated tau is toxic, whereas insoluble ubiquitinated phosphorylated tau is not toxic, and CHIP ubiquitinates 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

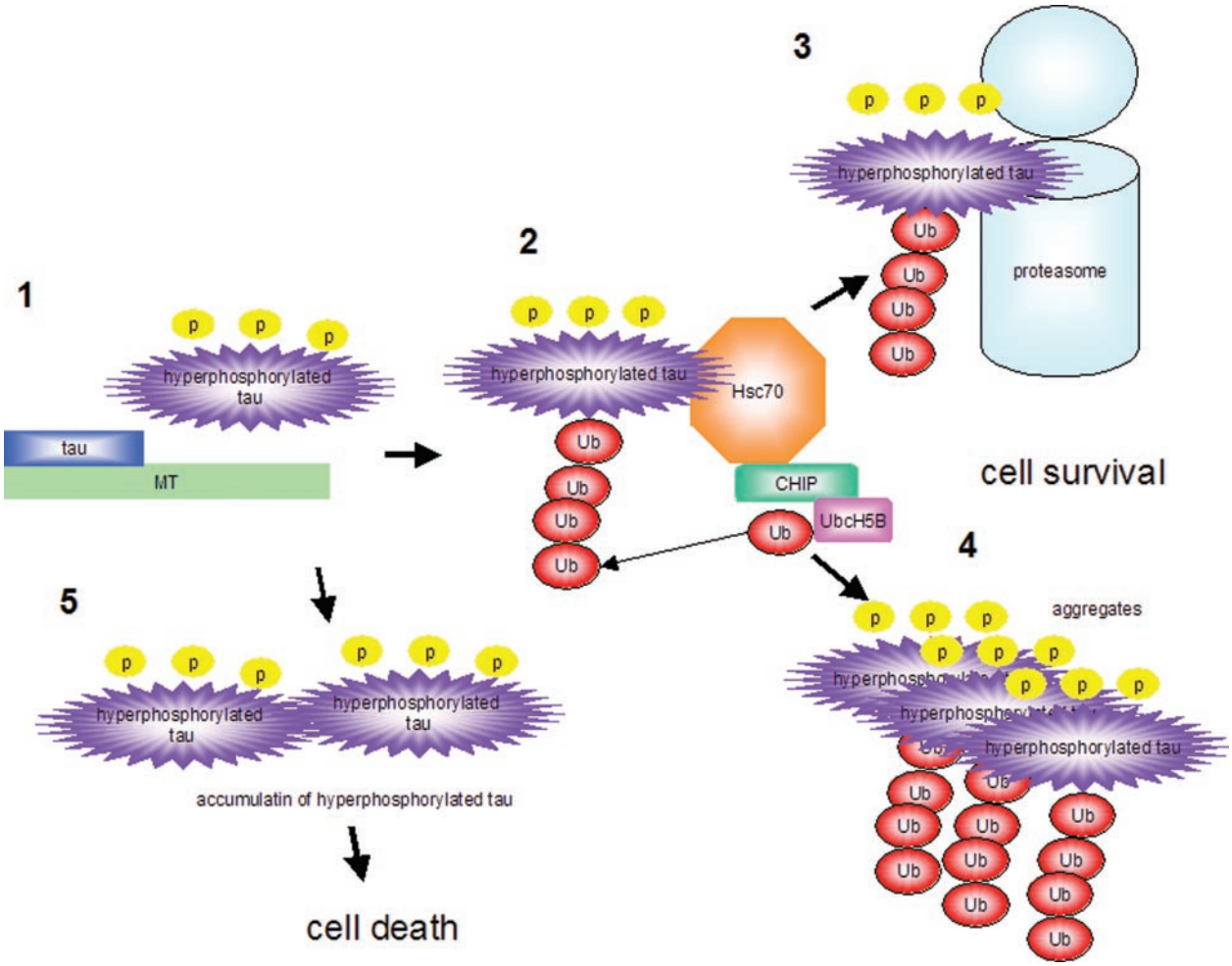

FIG. 6. **Model according to which the CHIP-Hsc70 complex decreases the toxicity of hyperphosphorylated tau.** *Step 1*, tau is phosphorylated by GSK-3β, Cdk5, and other kinases and thus released from microtubules. *Step 2*, hyperphosphorylated tau is ubiquitinated by the CHIP-Hsc70 complex for degradation to the proteasome (*step 3*) or formation of aggregates (*step 4*). *Step 5*, interference with step 2 leads to the accumulation of hyperphosphorylated tau.

1% Triton X-insoluble fraction. Consistent with a 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 additional accumulation of polyubiquitinated phosphorylated tau in the 1% Triton X-insoluble fraction of these cells did not severely enhance cell death. The data raise the possibility that insoluble ubiquitinated tau may not directly contribute to cell death.

EGFP-Tau Aggregates in Transfected Cells—To determine whether tau formed aggregates in the transfected cells, immunofluorescence staining was used to detect phosphorylated tau with AT8 or FLAG-Ub with the FLAG antibody. Filamentous green fluorescent bundles were observed in the cytoplasm of COS7 cells transfected with EGFP-tau, FLAG-Ub, Myc-CHIP, and mutant GSK-3β (Fig. 5*a*). Immunofluorescence staining using an anti-tubulin antibody showed that EGFP-tau co-localized to microtubules (data not shown). 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 (Fig. 5, *b* and *e*). The aggregates also stained with AT8 (Fig. 5, *c* and *d*) and anti-FLAG antibody (Fig. 5, *f* and *g*). These data indicate that ubiquitinated phosphorylated tau is a component of the aggregates. EGFP-tau appeared diffuse, and no aggregates containing EGFP-tau were found in cells transfected with GSK-3β, but not Myc-CHIP (Fig. 5*h*). Immunofluorescence staining of phosphorylated EGFP-tau using AT8 showed that phosphorylated EGFP-tau localized to the cytosol (Fig. 5, *i* and *j*). These cellular data agree with the biochemical findings in which insoluble ubiquitinated phosphorylated tau was found in cell lysates that expressed EGFP-tau, GSK-3β, FLAG-Ub, and Myc-CHIP.

DISCUSSION

We showed that phosphorylated tau was specifically ubiquitinated by CHIP complexed to Hsc70 in collaboration with UbcH5B. We developed a model in which phosphorylated tau induced cell death when tau and GSK-3β were exogenously expressed. CHIP expression attenuated the cell death caused by phosphorylated tau. Insoluble ubiquitinated phosphorylated tau and ubiquitinated phosphorylated tau-positive aggregates increased in the survivor cells, which exogenously expressed tau, CHIP, and GSK-3β. In contrast, neither U-box mutant CHIP nor the other ubiquitin ligases, parkin and Cbl, conjugated Ub onto hyperphosphorylated tau.

Recent studies have identified a co-chaperone protein, CHIP, whose three tetratricopeptide repeats bind to Hsc70/Hsp70 and Hsp90, whereas 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 (36), ErbB2 (37), the glucocorticoid receptor, and c-Raf kinase (38), were identified as target molecules of CHIP. Recently, Murata *et al.* reported that

heat-denatured luciferase was ubiquitinated by CHIP and proposed that it selectively ubiquitinates unfolded protein(s) by collaborating with molecular chaperones (39). Therefore, CHIP appears to play a role in targeting chaperone-protein complexes to the proteasome. The structural difference among the tau phosphoisoforms is unknown; however, certain tau phosphorylation events appear to trigger Hsc70 binding. Dou *et al.* (40) 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. CHIP and Hsc70 selectively recognized and ubiquitinated phosphorylated tau *in vivo* and *in vitro* in our study.

Intracellular protein aggregates underlie a variety of neurodegenerative diseases, such as Parkinson's disease, Huntington disease, fronto-temporal dementia, spino-cerebellar ataxias, and AD. All of these conditions present with intracellular protein aggregates, dominated by specific protein species, which are ubiquitinated. However, direct toxicity of the aggregates themselves has become a less attractive basis for explaining neuronal cell death in neurodegenerative diseases. In fact, partitioning an abnormally folded protein into an aggregate may actually forestall neurotoxicity. Support for this view comes from the loss of function of the Ub ligase, Ube3a in SCA1 mice, which increases neurodegeneration while decreasing the number of nuclear aggregates (19). Knock-in SCA1 mice form aggregates in the most vulnerable cells last and cells that are spared early on. In these models, it appears that sequestering of the aggregate can curtail toxicity. Instead, toxicity may arise from smaller assemblies in the form of oligomers or protofibrils. Here we showed that cells with exogenously expressed EGFP-tau, Myc-CHIP, GSK-3β, and FLAG-Ub have insoluble ubquitinated EGFP-tau aggregates and enhanced survival. In contrast, cells with exogenously expressed EGFP-tau, GSK-3β, and FLAG-Ub but lacked exogenous CHIP had no ubiquitinated EGFP-tau aggregates and reduced viability. These data indicate that Myc-CHIP conjugates Ub onto phosphorylated tau not only for degradation but also to form aggregates that may protect cells from the toxicity of soluble phosphorylated tau.

In summary, the CHIP-Hsc70 complex conjugates Ub to hyperphosphorylated tau in collaboration with UbcH5B. This reaction may also enhance cell survival by eliminating soluble phosphorylated tau in favor of insoluble aggregates (Fig. 6). As an endogenous mechanism for cell survival, the CHIP-Hsc70 complex may open new therapeutic approaches to AD and other neurodegenerative disorders.

Acknowledgments—We thank H. Band, J. Woodgett, and M. Medina for providing vectors and recombinant proteins. We also thank M. Schlossmacher for helpful comments and Jennifer Chan for helping us to obtain human brain tissue.

REFERENCES

1. Jellinger, K. A. (2003) *J. Neural Transm. Suppl.* **65,** 101–144
2. Lansbury, P. T., Jr., and Kosik, K. S. (2000) *Chem. Biol.* **7,** R9–R12
3. Kosik K. S. (1997) in *Brain Microtubule-associated Proteins*, pp. 43–52, Harwood Academic Publishers, Amsterdam
4. Johnson, G. V., and Jenkins, S. M. (1999) *J. Alzheimers Dis.* **1,** 307–328
5. Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A., and Hof, P. R. (2000) *Brain Res. Brain Res. Rev.* **33,** 95–130
6. Fath, T., Eidenmuller, J., and Brandt, R. (2002) *J. Neurosci.* **22,** 9733–9741
7. Ahlijanian, M. K., Barrezueta, N. X., Williams, R. D., Jakowski, A., Kowsz, K. P., McCarthy, S., Coskran, T., Carlo, A., Seymour, P. A., Burkhardt, J. E., Nelson, R. B., and McNeish, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2910–2915
8. Noble, W., Olm, V., Takata, K., Casey, E., Mary, O., Meyerson, J., Gaynor, K., LaFrancois, J., Wang, L., Kondo, T., Davies, P., Burns, M., Veeranna, Nixon, R., Dickson, D., Matsuoka, Y., Ahlijanian, M., Lau, L. F., and Duff, K. (2003) *Neuron* **38,** 555–565
9. Lucas, J. J., Hernandez, F., Gomez-Ramos, P., Moran, M. A., Hen, R., and Avila, J. (2001) *EMBO J.* **20,** 27–39
10. Spittaels, K., Van den Haute, C., Van Dorpe, J., Geerts, H., Mercken, M., Bruynseels, K., Lasrado, R., Vandezande, K., Laenen, I., Boon, T., Van Lint, J., Vandenheede, J., Moechars, D., Loos, R., and Van Leuven, F. (2000) *J. Biol. Chem.* **275,** 41340–41349
11. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998) *Nature* **392,** 605–608
12. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000) *Nat. Genet.* **25,** 302–305
13. Imai, Y., Soda, M., and Takahashi, R. (2000) *J. Biol. Chem.* **275,** 35661–35664
14. Zhang, Y., Gao, J., Chung, K. K., Huang, H., Dawson, V. L., and Dawson, T. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13354–13359
15. Maraganore, D. M., De Andrade, M., Lesnick, T. G., Farrer, M. J., Bower, J. H., Hardy, J. A., and Rocca, W. A. (2003) *Movement Disorders* **18,** 631–636
16. Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M. J., Jonnalagada, S., Chernova, T., Dehejia, A., Lavedan, C., Gasser, T., Steinbach, P. J., Wilkinson, K. D., and Polymeropoulos, M. H. (1998) *Nature* **395,** 451–452
17. McNaught, K. S., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C. W. (2003) *Exp. Neurol.* **179,** 38–46
18. De Vrij, F. M., Sluijs, J. A., Gregori, L., Fischer, D. F., Hermens, W. T., Goldgaber, D., Verhaagen, J., Van Leeuwen, F. W., and Hol, E. M. (2001) *FASEB J.* **15,** 2680–2688
19. Cummings, C. J., Reinstein, E., Sun, Y., Antalffy, B., Jiang, Y., Ciechanover, A., Orr, H. T., Beaudet, A. L., and Zoghbi, H. Y. (1999) *Neuron* **24,** 879–892
20. Rao, N., Dodge, I., and Band, H. (2002) *J. Leukocyte Biol.* **71,** 753–763
21. Shimura, H., Schlossmacher, M. G., Hattori, N., Frosch, M. P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K. S., and Selkoe, D. J. (2001) *Science* **293,** 263–269
22. Joachim, C. L., Morris, J. H., Selkoe, D. J., and Kosik, K. S. (1987) *J. Neuropathol. Exp. Neurol.* **46,** 611–622
23. Kosik, K. S., Orecchio, L. D., Binder, L., Trojanowski, J. Q., Lee, V. M., and Lee, G. (1988) *Neuron* **1,** 817–825
24. Biernat, J., Mandelkow, E. M., Schroter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Mercken, M., Vandermeeren, A., and Goedert, M. (1992) *EMBO J.* **11,** 1593–1597
25. Greenberg, S. G., Davies, P., Schein, J. D., and Binder, L. I. (1992) *J. Biol. Chem.* **267,** 564–569
26. Lee, V. M., Balin, B. J., Otvos, L., Jr., and Trojanowski, J. Q. (1991) *Science* **251,** 675–678
27. Lu, M., and Kosik, K. S. (2001) *Mol. Biol. Cell* **12,** 171–184
28. Gong, C. X., Wegiel, J., Lidsky, T., Zuck, L., Avila, J., Wisniewski, H. M., Grundke-Iqbal, I., and Iqbal, K. (2000) *Brain Res.* **853,** 299–309
29. Sontag, E., Nunbhakdi-Craig, V., Lee, G., Brandt, R., Kamibayashi, C., Kuret, J., White, C. L., 3rd, Mumby, M. C., and Bloom, G. S. (1999) *J. Biol. Chem.* **274,** 25490–25498
30. Papasozomenos, S. C., and Su, Y. (1995) *J. Neurochem.* **65,** 396–406
31. Sontag, E., Nunbhakdi-Craig, V., Lee, G., Bloom, G. S., and Mumby, M. C. (1996) *Neuron* **17,** 1201–1207
32. Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K., and Ihara, Y. (1995) *J. Biol. Chem.* **270,** 823–829
33. Illenberger, S., Zheng-Fischhofer, Q., Preuss, U., Stamer, K., Baumann, K., Trinczek, B., Biernat, J., Godemann, R., Mandelkow, E. M., and Mandelkow, E. (1998) *Mol. Biol. Cell* **9,** 1495–1512
34. Jiang, J., Ballinger, C. A., Wu, Y., Dai, Q., Cyr, D. M., Hohfeld, J., and Patterson, C. (2001) *J. Biol. Chem.* **276,** 42938–42944
35. Dominguez, I., Itoh, K., and Sokol, S. Y. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8498–8502
36. Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr, D. M. (2001) *Nat. Cell Biol.* **3,** 100–105
37. Zhou, P., Fernandes, N., Dodge, I. L., Reddi, A. L., Rao, N., Safran, H., DiPetrillo, T. A., Wazer, D. E., Band, V., and Band, H. (2003) *J. Biol. Chem.* **278,** 13829–13837
38. Demand, J., Alberti, S., Patterson, C., and Hohfeld, J. (2001) *Curr. Biol.* **11,** 1569–1577
39. Murata, S., Minami, Y., Minami, M., Chiba, T., and Tanaka, K. (2001) *EMBO Rep.* **2,** 1133–1138
40. Dou, F., Netzer, W. J., Tanemura, K., Li, F., Hartl, F. U., Takashima, A., Gouras, G. K., Greengard, P., and Xu, H. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 721–726