Akt and CHIP coregulate tau degradation through coordinated interactions

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A hallmark of the pathology of Alzheimer’s disease is the accumulation of the microtubule-associated protein tau into fibrillar aggregates. Recent studies suggest that they accumulate because cytosolic chaperones fail to clear abnormally phosphorylated tau, preserving a pool of toxic tau intermediates within the neuron. We describe a mechanism for tau pathology involving a major cellular kinase, Akt. During stress, Akt is ubiquitinated and degraded by the tau ubiquitin ligase CHIP, and this largely depends on the Hsp90 complex. Akt also prevents CHIP-induced tau ubiquitination and its subsequent degradation, either by regulating the Hsp90/CHIP complex directly or by competing as a client protein with tau for binding. Akt levels tightly regulate the expression of CHIP, such that, as Akt levels are suppressed, CHIP levels also decrease, suggesting a potential stress response feedback mechanism between ligase and kinase activity. We also show that Akt and the microtubule-affinity-regulating kinase 2 (PAR1/MARK2), a known tau kinase, interact directly. Akt enhances the activity of PAR1 to promote tau hyperphosphorylation at S262/S356, a tau species that is not recognized by the CHIP/Hsp90 complex. Moreover, Akt1 knockout mice have reduced levels of tau phosphorylated at PAR1/MARK2 consensus sites. Hence, Akt serves as a major regulator of tau biology by manipulating both tau kinases and protein quality control, providing a link to several common pathways that have demonstrated dysfunction in Alzheimer’s disease.

Alzheimer’s disease | cell signaling | chaperone | MARK2/PAR1

The master cellular kinase Akt has also been established as a client of the Hsp90 complex, and it can be degraded by inhibiting Hsp90 ATPase activity with small molecules aptly named Hsp90 inhibitors (5–7); however, the mechanism of degradation and the involvement of CHIP in this process have yet to be determined. Moreover, as many of the proteins comprising the Hsp90 complex are phosphorylated directly, we reasoned that, although Akt may indeed be a client of the Hsp90/CHIP complex, perhaps Akt was also regulating its activity during these periods of interaction and consequently may impact tau biology in a distinct and novel way. Abnormal tau accumulation results from hyperphosphorylation of tau by a number of kinases, Akt included; however, regulation of tau degradation by Akt and the Hsp90/CHIP complex may play an even greater role in the abnormal accumulation that occurs with diseases of aging. Establishing such a relationship would also demonstrate a functional consequence of a kinase/chaperone signaling network that could be involved in the regulation of a number of other important proteins within the cell. Here we investigated whether CHIP, acting in concert with Hsp90, could promote Akt degradation. We also explored whether Akt could regulate the activity of the CHIP/Hsp90 complex, and we examined what impact this regulation might have on the activity of the chaperone complex to facilitate tau degradation, particularly with regard to degradative-resistant tau phosphorylated by PAR1/MARK2.

Results

Ablation of CHIP Leads to Increased Akt in Mouse Brain. Basso et al. (5) found that, when Hsp90 was inhibited, Akt levels decreased, and we confirmed that result using the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) in cells over-expressing Akt1 (Fig. 1A). Targeted knockdown of either CHIP or Hsp90α with siRNA (previously characterized (3)) attenuated 17-AAG-mediated degradation of Akt (Fig. 1A). As expected, 17-AAG increased the levels of both Hsp70 and Hsp90, whereas cells treated with CHIP siRNA had reduced basal Hsp70 levels (8). Knockdown of CHIP and Hsp90α also increased endoge-


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A result of aberrant kinase activation or stress-associated activity was indeed due to the absence of CHIP and not simply from CHIP or activity (SI Fig. 7). Mice, the Hsp90 complex is necessary for homeostatically regulating levels of phospho-GSK3β. Moreover, Akt’s major cellular target, glycogen synthase kinase 3β (GSK3β), also exhibited significantly elevated levels in CHIP knockout mice and after a stress response, we wanted to investigate the consequence of reducing Akt levels on CHIP, perhaps providing evidence to support Akt as a critical stress mediator. To determine whether CHIP promotes Akt ubiquitination in cell culture systems, Cells were cotransfected with WT Akt and myc-CHIP for 24 h in the presence or absence of the proteasomal inhibitor epoxomicin for 6 h. CHIP coimmunoprecipitation showed enhanced CHIP/Akt complexes and ubiquitination of Akt in the presence of epoxomicin. (D) Cells transfected with CHIP or control siRNA for 72 h were then treated with 0.5 μM 17-AAG and 50 μM cycloheximide, and cells were harvested at indicated time points. CHIP siRNA prevented endogenous Akt degradation.

Hsp90 (SI Fig. 8A). The results confirmed that the CHIP/Akt interaction was largely dependent on Hsp90 or Hsp70, because mutated CHIP formed fewer complexes with Akt than WT CHIP (Fig. 2B).

Based on that evidence, we determined whether CHIP promotes Akt ubiquitination in cell culture systems. Cells were cotransfected with WT Akt and myc-CHIP for 24 h in the presence or absence of the proteasomal inhibitor epoxomicin for 6 h. Coimmunoprecipitation of Akt revealed increased levels of polyubiquitinated Akt in cells cotransfected with CHIP (Fig. 2C); however, the amount of ubiquitin immunoreactivity markedly increased in the presence of the inhibitor, suggesting that Akt is processed in a proteasomal-dependent manner.

Next we determined whether CHIP is involved in the protein turnover of endogenous Akt. As shown in Fig. 2D, cycloheximide pulse–chase experiments showed that degradation of endogenous Akt decreased in Hs735 and Hs587 cells transfected with CHIP siRNA in the presence of 17-AAG compared with cells transfected with control siRNA and 17-AAG (Fig. 2D). Conversely, CHIP overexpression enhanced the 17-AAG-mediated degradation of overexpressed Akt (SI Fig. 8B). We also determined that CHIP interacted with Akt independent of its phosphorylation status because both WT Akt and mutant Akt mock-phosphorylated at the T308 and S473 sites bound to CHIP (SI Fig. 8C). Together these results show that CHIP ubiquitinates and degrades Akt; however, this depends on the presence of Hsp90 and perhaps attenuated Hsp90 ATPase activity.

CHIP Levels in Akt1−/− Brain. Previous work has suggested that the PI3K/Akt cascade is involved in the transactivational regulation of the heat shock response (12, 13). Because CHIP maintains homeostasis by regulating the degradation of proteins during and after a stress response, we wanted to investigate the consequence of reducing Akt levels on CHIP, perhaps providing evidence to support Akt as a critical stress mediator. To deter-

**Fig. 1.** The CHIP/Hsp90 complex is required for Hsp90 inhibitor-mediated Akt degradation. (A) HeLa cells transfected with siRNA pools for Hsp90α, CHIP, or a nonsilencing control were then transfected with WT Akt and treated with 0.5 μM 17-AAG for an additional 24 h. IRSAs for both CHIP and Hsp90α prevented Akt degradation. (B) Brain homogenates from eight mice (four CHIP−/− and four CHIP+/+) were analyzed by Western blot using the indicated antibodies. Total Akt (P = 0.0029) and p59 Gsk3β (P = 0.007) protein levels were significantly decreased (27 ± 7% and 39 ± 8%) in CHIP−/− mice compared with CHIP+/+ littermates after normalization to GAPDH and total GSK3β, respectively (SI Fig. 7B).

To validate that this marked elevation in Akt levels and activity was indeed due to the absence of CHIP and not simply a result of aberrant kinase activation or stress-associated activities of the CHIP−/− mice, we examined levels of Akt in another disease mouse model, twitcher, which has a similar phenotype caused by a naturally occurring nonsense mutation in the GALC (galactosylceramidase) gene (11). Unlike the CHIP knockout mice, the twitcher mice had no evidence of increased Akt levels or activity (SI Fig. 7C). These results indicate that the CHIP/Hsp90 complex is necessary for homeostatically regulating levels of Akt in the brain.

**Interaction of Akt with CHIP in Brain Tissue.** We then investigated the physiological interaction between Akt and CHIP in vivo. We coimmunoprecipitated CHIP from whole-brain homogenates of Akt1−/− and Akt1+/+ mice and determined that CHIP does bind to Akt1 (Fig. 2A). To check whether Hsp90 mediates the CHIP/Akt interaction, we used CHIP harboring the K30A mutation, which prevents CHIP from binding to Hsp70 and

**Fig. 2.** CHIP binds Akt in a predominantly Hsp70/90-dependent manner, promoting its ubiquitination and degradation regardless of phosphorylation state. (A) CHIP coimmunoprecipitation from brain lysates of Akt1−/− and Akt1+/+ mice showed CHIP binding to endogenous Akt1 only in WT (+/+ ) mice. (B) Hek293 cells were transfected with HA-tagged WT Akt and myc-tagged WT CHIP, myc-CHIP harboring the K30A mutation, or empty vector. Akt coimmunoprecipitation showed that the K30A mutation in CHIP abrogated Akt/CHIP complex formation. (C) Cells were transfected with WT Akt and ubiquitin and either myc-CHIP or empty vector. After 24 h, 1 μM epoxomicin was added to media for 6 h. Akt coimmunoprecipitation showed enhanced CHIP/Akt complexes and ubiquitination of Akt in the presence of epoxomicin. (D) Cells transfected with CHIP or control siRNA for 72 h were then treated with 0.5 μM 17-AAG and 50 μM cycloheximide, and cells were harvested at indicated time points. CHIP siRNA prevented endogenous Akt degradation.
Akt siRNA Accelerates tau Degradation by Inhibiting Hsp90. Given this novel interaction between Akt and CHIP in conjunction with the role of CHIP in contextual substrate-dependent stress recovery (1), we determined whether the presence of another CHIP substrate, in this case Akt, interferes with CHIP-mediated tau ubiquitination and degradation. First, we reduced Akt levels by siRNA and inhibited PI3K activity using LY294002 to determine how that would affect Hsp90 inhibitor-mediated tau degradation in cell culture. Consistent with our previous findings, we observed a dose-dependent decrease in phospho-tau levels after treatment with 17-AAG (4, 15). Both treatment with Akt siRNA (SI Fig. 10A) and exposure to LY294002 reduced 17-AAG-mediated induction of the heat shock proteins, although LY294002 had a much more pronounced effect (Fig. 4A and SI Fig. 10B). However, only Akt siRNA accelerated Hsp90 inhibitor-mediated tau degradation, suggesting that this process did not depend on HSF1 activation (Fig. 4A).

Using coimmunoprecipitation, we found that, when we reduced Akt levels with siRNA, CHIP binding to and ubiquitination of tau was significantly enhanced (Fig. 4B). We also found that Akt and CHIP could independently and concomitantly bind tau, but the number of CHIP/tau complexes was reduced in the presence of Akt overexpression (SI Fig. 11). Coimmunoprecipitated CHIP/tau complexes from our cell culture model were then analyzed by Western blot for total Akt levels, along with CHIP and HSF1. Akt siRNA that targeted Akt1 (optimized in SI Fig. 10A) reduced both CHIP protein (Fig. 3B; second cell line in SI Fig. 9B) and mRNA levels (Fig. 3C), as determined by Western blot and real-time PCR, respectively. Surprisingly, overexpression of the heat shock factor 1 (HSF1) or forkhead transcription factor 3A (FOXO3A), both of which are regulated upstream by the PI3K/Akt cascade, failed to reverse CHIP reductions induced by Akt siRNA (SI Fig. 9C). When Akt activity was inhibited with LY294002 or overexpressed with constitutive active Akt, neither CHIP protein (SI Fig. 9D) nor mRNA level (data not shown) was affected, indicating that CHIP expression is tied to Akt levels, but only to a threshold. These data suggest the presence of a feedback system based on Akt levels, not activity, that regulates the expression of CHIP through a novel signaling pathway.

Akt siRNA and PI3K/Akt activity regulate CHIP expression. Previous studies show that the presence of CHIP, which is required for Hsp90 function, is regulated by Akt activity. Given this, we tested whether coimmunoprecipitation revealed that Akt siRNA enhanced CHIP binding and ubiquitination of tau. (C) Cells were transfected with myc-CHIP and V5-tau or empty vector. CHIP/tau complexes were coimmunoprecipitated with an anti-myc antibody and incubated with 100 ng of recombinant Akt (rAkt) for 16 h. After washing, Western blot showed less tau bound to CHIP in those lysates incubated with rAkt. Inputs confirm the presence of rAkt in the unbound fraction.

Akt Enhances PAR1-Mediated Phosphorylation of tau. We previously showed that tau phosphorylated by PAR1/MARK2 could not be

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recognizes the τ Tau phosphorylation events S262/S356, which are not recognized by CHIP or degraded by Hsp90 inhibition (3, 4). The individual relationships between Akt and GSK3β and between PAR1/MARK2 and GSK3β, we wanted to investigate the potential interaction among all three of these proteins, particularly in the context of how their interaction affects tau degradation. Cells were cotransfected with WT Akt, myc-PAR1, or both for 48 h. Akt coimmunoprecipitation and myc immunoblotting showed that PAR1 bound to Akt. Reverse coimmunoprecipitation with myc demonstrated Akt binding with overexpressed Act. Inputs refer to cell lysates before coimmunoprecipitation. (C Upper) Cells were transfected with WT Akt and myc-PAR1 together with either WT Akt or CA Akt for 48 h and analyzed by Western blot. Tau phosphorylated at S396/S404 was detected only in cells cotransfected with each Akt plasmid and PAR1. Total tau levels were also elevated (V5). Tau phosphorylated at S262/S356 was detectable only in cells transfected with PAR1. GAPDH was used for loading normalization. (C Lower) Cells were transfected with V5-tau and PAR1 alone, or PAR1 together with either WT Akt or CA Akt for 48 h and analyzed by Western blot. Tau phosphorylated at S396/S404 was detectable in cells cotransfected with each Akt plasmid and PAR1. Total tau levels were also elevated (V5). Tau phosphorylated at S262/S356 was detectable only in cells transfected with PAR1. GAPDH was used for loading normalization. (D) Brain homogenates from six mice (three Akt1−/− and three Akt1+/+) were analyzed by Western blot using the indicated antibodies. Deletion of Akt1 was confirmed, and tau phosphorylated at S262/S356 was significantly lower in Akt1−/− mice. Total tau and GAPDH levels were unchanged.

Discussion

Because hyperphosphorylated tau pathology is present in the brains of AD patients, the role of kinases in the pathogenesis of AD has been studied extensively during the past two decades (17). Here we propose a role for Akt in AD pathogenesis, distinct from its kinase activity (Fig. 6). Our results suggest that Akt regulates the CHIP/Hsp90 complex; this role appears to have a greater impact on tau accumulation than phosphorylation. CHIP ubiquitinates and degrades Akt, particularly when Hsp90 ATPase activity is inhibited. Akt reduced CHIP-mediated tau ubiquitination and slowed its degradation. We also found a link between Akt and the critical tau kinase PAR1/MARK2, whereby Akt enhances phosphorylation of tau at S262/S356, a site that is not recognized by the CHIP/Hsp90 degradation complex. In
and pGSK3
CHIP as a critical component of this process (Fig. 1B). In fact, CHIP−/− mouse brain homogenates had increased levels of Akt and pGSK3β Ser-9, further suggesting the critical interaction between these two proteins (Fig. 1A). Although we have identified the mechanism of their interaction, we can only speculate on its purpose. For example, CHIP−/− mice have increased apoptotic activity throughout their bodies despite having elevated prosurvival Akt gene expression (2, 8).

Therefore, Akt and CHIP might cooperate to balance apoptotic signaling. As Akt levels decrease, CHIP expression levels also decrease, promoting apoptosis (Fig. 3); however, as Akt levels increase, CHIP regulates Akt prosurvival activity by facilitating its degradation. Qian et al. (1) recently demonstrated a similar scenario for Hsp70, whereby CHIP maintained Hsp70 levels based on the cellular context. Thus, CHIP balances proteins that have a major impact on the cellular environment; however, if these protein levels fall below an acceptable limit, CHIP must be down-regulated to prevent further decreases. There also appears to be a threshold for CHIP levels, such that, despite Akt overexpression, CHIP remains constant, perhaps providing additional information about the mechanism of Akt oncogenesis: Akt levels within the cell might exceed the capacity of CHIP. Conversely, Akt siRNA dramatically reduced CHIP but only modestly affected Hsp induction. Because neither HSF1 nor FOXO3A rescued CHIP expression from Akt knockdown, we propose that a distinct pathway directly mediated by Akt is acting on the STUB1 (CHIP) promoter. Moreover, the disparity between LY294002-mediated and Akt siRNA-mediated Hsp induction suggests that a component of PI3K signaling that is distinct from the classical Akt cascade dramatically alters HSF1 activity.

Based on our previous work, which defined phospho-tau as a client of the Hsp90/CHIP complex (3, 4), we hypothesized that Akt has regulatory effects on the phospho-tau/CHIP interaction. Indeed, we found that tau degradation by Hsp90 inhibition was enhanced only when the endogenous Akt level, not Akt activity, was reduced (Fig. 4A). CHIP ubiquitination of Akt was also enhanced when Akt levels were reduced (Fig. 4B). Hence, Akt might regulate the Hsp90/CHIP complex by impairing or slowing the degradation of its anti-tau activity, and this regulation might occur by directly modifying the client-bound complex itself, competing with other substrates, or modifying the tau protein. The evidence we presented suggests that competition for CHIP binding plays a role. For example, when we overexpressed Akt, the number of CHIP/tau complexes decreased (SI Fig. 11A), and recombinant Akt abrogated the CHIP–tau interaction (Fig. 4C).

This idea of substrate competition would be supported by work showing that CHIP-mediated stress recovery occurred by sequential ubiquitination of substrates and Hsp70 (1). However, given the role of Akt as a kinase and modulator of Hsp90 itself, along with our findings that Akt directly binds to tau (SI Fig. 11), multiple Akt-mediated mechanisms could be involved in the process of tau degradation. Our findings suggest that Akt be considered in a novel context with regard to AD; rather than strictly serving as a modifier of tau phosphorylation, Akt likely serves a much more important role as a regulator of tau degradation.

Another way in which Akt indirectly modifies tau biology is through the impact that it has on tau phosphorylation by other kinases, particularly given this new function for Akt as a negative regulator of tau degradation. We demonstrated that tau phos-

phorylated by PAR1/MARK2 at S262/S356 was unrecognizable to CHIP (3, 4). Furthermore, Nishimura et al. (16) showed that PAR1/MARK2 might nucleate tau hyperphosphorylation by priming it for other kinases. Indeed, Akt significantly enhanced the acetylation of phospho-tau in combination with either GSK3β or PAR1/MARK2, suggesting that Akt abrogates tau degradation and provides a higher concentration of exposed substrate for other tau kinases.

Perhaps the most intriguing result was the combined effect of Akt and PAR1/MARK2 on tau, namely, increased phosphorylation of tau at epitopes other than those typically phosphorylated by PAR1/MARK2 (16). This suggested that Akt either increased tau levels by preventing degradation, allowing PAR1/MARK2 to prime tau for other endogenous kinases, or modified PAR1/MARK2 activity directly. Indeed we found that Akt and PAR1/MARK2 interact directly, suggesting that one might regulate the other’s activity. Using Akt−/− mice, we also found that pS262/S356 tau was selectively reduced compared with total tau levels (Fig. 5D). This further implied a direct and functional interaction between Akt and PAR1/MARK2 in the brain, one in which Akt regulated PAR1/MARK2 activity. However, this could also indicate the significance of Akt in regulating the CHIP/Hsp90 complex with regard to tau biology. As Akt levels were depleted, not only did PAR1/MARK2 activity decrease, but tau degradation also became enhanced. This might bridge PAR1/MARK2 with the CHIP/Hsp90 complex to prevent tau degradation on multiple fronts. Moreover, although the impact of this Akt/PAR1/MARK2 complex on the CHIP/Hsp90 complex itself is not known, the implications are far-reaching for AD research given the nature of the signaling cascades in which these kinases are involved and their link with AD pathogenesis.

Materials and Methods

Materials. Akt1−/− mouse tissues were obtained from M.J.B. CHIP−/− mice were originally obtained from C.P.

Plasmids. PAR1 was provided by Bing-Wei Lu. Constitutively active GSK3β was provided by Ben Wolozin. Myc-CHIP, V5-tau, and HA-ubiquitin were generated by our laboratory. All Akt constructs were provided by J.O.C. Myc-CHIP K30A mutant was provided by C.P. HSF1 plasmid was provided by Richard Voellmy. FOXO3A plasmid was provided by Wenleng Bai.

Antibodies, siRNAs, and Chemicals. 12E8 (anti-pS262/S356 tau) was provided by Peter Seubert. PHF1 (anti-pS396/404) and CP1 (pS202/T205 tau) were provided by Peter Davies. Tau 5 (anti-total tau) was provided by Lester Binder. Anti-Actin was obtained from Invitrogen. Anti-myc and anti-HA were obtained from Invitrogen. Anti-Hsp70 and HSP90α were obtained from Stressgen. Anti-GAPDH was obtained from Biodiesis. Anti-Akt, Akt1, GSK3β, and GSK3β Ser-9 were obtained from Cell Signaling Technology. Anti-CHIP and E1 were generated by our group. Secondary antibodies were obtained from Southern Biotechnology Associates and Jackson Immunonochemicals. All antibodies were used at a 1:1,000 dilution, except for PHF1 and CP13, which were used at 1:100, and V5, which was used at 1:5,000. All siRNAs were obtained from Qiagen and were previously described and characterized at 20 nM final concentration (3). Akt siRNAs were validated by Qiagen (Akt1-5HP and Akt1-10HP). 17-AAG (500 nM) was obtained from A.G. Scientific. LY294002 (100 μM) and epoxomicin (100 nM) were obtained from Calbiochem. Cycloheximide (50 μM) was obtained from Sigma–Aldrich.

Mouse Tissue. Akt1−/− brain tissue was provided by M.J.B. CHIP−/− mice were generated by C.P. and bred at the Mayo Clinic.

Cell Culture and Transfections. HEla and HEK293 cells were grown in Opti-Mem plus 10% FBS and passaged every 3–5 days based on 90% confluence. HS78T cells were grown in Leibovitz medium with 10% FBS. siRNA and communo-
precipitation experiments were carried out as previously described (2, 3).

Communoprecipitations and Western Blotting. Akt and tau ubiquitination levels in HEla and HEK293 cells was performed as previously described (18, 19). Briefly, cell supernatants were incubated with capture antibodies and Fc binding protein overnight at 4°C. Supernatants were washed and subjected to
Western blot analyses after SDS/PAGE electrophoresis. Blots were probed with indicated antibodies.

**Brain Tissue Preparation.** Frozen on dry ice for subsequent biochemical analyses. Mice lacking CHIP or Akt1 expression (CHIP−/−, Akt1−/−) were generated as previously described (8, 20). CHIP−/− and CHIP+/+ mice were humanely killed at postnatal day 30, and brains were quickly removed for dissection and frozen on dry ice for subsequent biochemical analyses. Similarly, Akt1−/− and Akt1+/+ mice were humanely killed at 3 months of age, and tissues were harvested.

**Quantitative Real-Time PCR.** Real-time PCR was performed as previously described (21). Briefly, total RNA was prepared by using the RNeasy kit (Qiagen) and reverse-transcribed by using SuperScript II (Invitrogen). cDNA was amplified by using 2× SYBR green master mix (Applied Biosystems) and analyzed on the Bio-Rad Opticon.

**Statistical Analyses.** For cell culture and animal tissue experiments, Student's t test was used to assess significance, where reductions or increases in protein levels were demonstrated by Western blot. GAPDH immunoreactivity was used to normalize for lane-to-lane variation. All replicates within each experiment and the values from subsequent replicate experiments were included in the analyses.

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