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The Peptidylprolyl *cis/trans*-Isomerase Pin1 Modulates Stress-induced Dephosphorylation of Tau in Neurons

*IMPLICATION IN A PATHOLOGICAL MECHANISM RELATED TO ALZHEIMER DISEASE**

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Deregulation of Tau phosphorylation is a key question in Alzheimer disease pathogenesis. Recently, Pin1, a peptidylprolyl *cis/trans*-isomerase, was proposed to be a new modulator in Tau phosphorylation in Alzheimer disease. *In vitro*, Pin1 was reported to present a high affinity for both Thr(P)-231, a crucial site for microtubule binding, and Thr(P)-212. In fact, Pin1 may facilitate Thr(P)-231 dephosphorylation by protein phosphatase 2A through trans isomerization of the Thr(P)-Pro peptide bound. However, whether Pin1 binding to Tau leads to isomerization of a single site or of multiple Ser/Thr(P)-Pro sites *in vivo* is still unknown. In the present study, Pin1 involvement was investigated in stress-induced Tau dephosphorylation with protein phosphatase 2A activation. Both oxidative (H_2O_2) and heat stresses induced hypophosphorylation of a large set of phospho-Tau epitopes in primary cortical cultures. In both cases, juglone, a Pin1 pharmacological inhibitor, partially prevented dephosphorylation of Tau at Thr-231 among a set of phosphoepitopes tested. Moreover, Pin1 is physiologically found in neurons and partially co-localized with Tau. Furthermore, in Pin1-deficient neuronal primary cultures, H_2O_2 stress-induced Tau dephosphorylation at Thr(P)-231 was significantly lower than in wild type neurons. Finally, Pin1 transfection in Pin1-deficient neuronal cell cultures allowed for rescuing the effect of H_2O_2 stress-induced Tau dephosphorylation, whereas a Pin1 catalytic mutant did not. This is the first demonstration of an *in situ* Pin1 involvement in a differential Tau dephosphorylation on the full-length multiphosphorylated substrate.

In Alzheimer disease (AD)[2] brain, Tau is the main component of the paired helical filaments that compose neurofibrillary tangles, a hallmark of the disease. The phosphoprotein Tau is a key regulator of microtubule dynamics. The functions of Tau are regulated by site-specific phosphorylation events (1). Among the numerous phosphorylation sites in Tau, Thr-231 plays a critical regulatory role because its phosphorylation greatly diminishes the ability of Tau to bind and stabilize microtubules in the cell (2–4). In neurofibrillary tangles, Tau is hyperphosphorylated on many serine or threonine residues preceding proline (Ser/Thr(P)-Pro). Among them, Thr-212 and Thr-231 are Tau conformation-dependent epitopes clearly identified as abnormally phosphorylated in AD (5, 6).

A complex set of modulators tightly regulates the dynamic phosphorylation state of Tau. The phosphorylation state of a protein reflects the balance between activation and inhibition of kinases, phosphatases, and partners able to support their activity through conformational changes. A deregulation of these mechanisms is a central point in the etiopathology of AD and other neurodegenerative pathologies (for review Ref. 1). Thus, characterization of proteins that modulate Tau phosphorylation-dephosphorylation processes is essential for the understanding of the pathological events. The peptidylprolyl isomerase Pin1 catalyzes the cis/trans isomerization in a subset of proteins on Ser(P)/Thr(P) residues preceding proline. Although Pin1 has been widely studied in the cell cycle and cancer, little is known about its role in the brain. Recent data suggested that Pin1 might be involved in AD pathogenesis, because it is found in neurofibrillary tangles (7). *In vitro* studies showed that Pin1 was able to bind Tau through Thr(P)-231 (7). Nevertheless, further studies on Pin1 knock-out mice suggested that there was an inverse correlation between Pin1 expression and neurofibrillary degeneration in AD (8). Recently, NMR studies reported a high affinity for both Thr-231 and Thr-212 of Tau on Pin1 (9, 10). Deregulation of Pin1 activity, expression or localization might induce an imbalance in the phosphorylation-dephosphorylation state at specific epitopes inducing a disturbance in the interactions between Tau and its target proteins. In fact, Pin1 may facilitate Tau dephosphorylation by protein phosphatase 2A (PP2A) (11). However, the mechanisms underlying the functional involvement of Pin1 in Tau deregulation are still unknown.

Oxidative stress, an early event in Alzheimer disease occurring prior to cytopathology, induces rapid hypophosphorylation of Tau epitopes (12). This Tau dephosphorylation is likely to be mediated through PP2A activation (13). Such PP2A activation leading to Tau dephosphorylation is also observed in other stress conditions such as heat shock (13, 14). In the present study, the potential involvement of Pin1 in Tau dephosphorylation was assessed in primary neuronal cell cultures using

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[2] The abbreviations used are: AD, Alzheimer disease; PP2A, protein phosphatase type 2A; Pin1, peptidylprolyl *cis/trans*-isomerase (EC 5.2.1.8); juglone, 5-hydroxy-1,4-naphthoquinone; PIPES, 1,4-piperazinediethanesulfonic acid.

both oxidative and heat stresses and a pharmacological Pin1 inhibitor, juglone (15). The role of Pin1 was further investigated in Pin1-deficient cultures obtained from Pin1 knock-out mice embryos. In these studies, we demonstrated that Pin1 plays an active role in stress-induced Tau dephosphorylation, preferentially at Thr-231 epitope. This is the first demonstration of an *in situ* Pin1 involvement in a differential Tau dephosphorylation.

EXPERIMENTAL PROCEDURES

Primary Embryonic Neuronal Culture—Rat primary cortical neurons were prepared from 17–18-day-old Wistar rat embryos as follows. Briefly, brain and meninges were removed. Cortex was carefully dissected out and mechanically dissociated in culture medium by triturating with a polished Pasteur pipette. Once dissociated and after blue trypan counting, cells were plated at a density of 2.5×10^4 cells/cm^2 in poly-D-lysine and laminin-coated 24-well plates or Labtech (Becton Dickinson). For dissociation, plating, and maintenance, we used Neurobasal medium supplemented with 2% B27 containing 200 mM glutamine and 1% antibiotic-antimycotic agent (Invitrogen). Medium was replenished every 3 days and 24 h before cell treatment.

Pin1 Knock-out Primary Embryonic Neuronal Culture—For knock-out studies, primary cortical neurons were prepared from 15–17-day-old mice embryos. Pin1-deficient neurons were obtained from embryos derived from heterozygous pairings. Homozygous pairings are not possible because homozygous male are sterile. Each embryo was dissected and plated individually. In each experiment, neurons acquired from wild type littermate embryos were used as controls. The genotype of each embryo was determined using the remaining tissue and primers as described in Fujimori *et al.* (16).

Pin1 cDNAs—Pin1 cDNA was obtained from Prof. P. Davies (AECOM, Bronx, NY). It was subcloned in a pcDNA4 plasmid (Invitrogen). Site-directed mutagenesis was made by PCR using the QuikChange system from Stratagene. Ser-115, a residue located close to the juglone targeted Cys-113, was mutated into glutamate. It is a consensus residue among peptidylprolyl isomerases and breaks one of the helixes of the catalytic pocket (17). Pin1 S115E sequence primers were sense, 5′-CAGCGACTGCAGC**GA**AGCCAAGGCCAGG-3′ and anti-sense, 5′-CCTGGCCTTGGCTTCGCTGCAGTCGCT-3′. These three pcDNA4 plasmids (empty, Pin1, and [S115E]Pin1) were used for further transfections.

Transfection of Primary Embryonic Culture—Primary cortical cultures were transfected 7 days after plating with Pin1 cDNA plasmids using a modified Ca^{2+}-phosphate transfection method (18). Briefly, culture medium was replaced by prewarmed Dulbecco's modified Eagle's medium and preserved. Both the original culture medium and cells were returned to a 5% CO_2 incubator. In the meantime, the DNA/Ca^{2+}-phosphate precipitate was prepared using ProFection Mammalian Transfection System (Promega). Per well (24-well plate), 3 μg of cDNA and 3.1 μl of 2 M $CaCl_2$ adjusted with H_2O to a total volume of 25 μl (solution A) were mixed to 25 μl of 2 × Hanks' balanced salt (solution B). Solution A was added by ~1/8 volume to solution B while gently mixing (600 rpm). The mixed solution was kept at −20 °C for 5 min to prime the precipitate and then at room temperature for 15 min. The DNA/Ca^{2+}-phosphate suspension solution was added dropwise to each well. Cells were incubated in presence of the precipitate for 30 min in a 5% CO_2 culture incubator at 37 °C. After incubation, transfection medium was replaced by 10% CO_2-equilibrated Dulbecco's modified Eagle's medium and incubated for 20 min in a 5% CO_2 incubator. Dulbecco's modified Eagle's medium was replaced by the original culture medium, and cells were returned to 5% CO_2 incubator.

Cell Treatment—At 10 days *in vitro*, cells were preincubated for 2 h with 1 μM juglone and/or treated with 1 mM H_2O_2 for the indicated time, or exposed to 44 °C in a 5% CO_2 incubator for 30 min. Thirty percent stock hydrogen peroxide (Prolabo) was dissolved in 0.2 M phosphate-buffered saline (pH 7.4). Thirty-one μM stock okadaic acid (Sigma) was dissolved in ethanol.

COS Cell Culture—COS cells were grown as described previously (19). Transient transfection was performed using ExGen (Euromedex, France), and cell lysates were analyzed after 24 h (2, 19, 20).

Antibodies—AD2 is directed against phosphorylated Ser-396 and Ser-404, Tau1 recognizes Tau only when serines 195, 198, 199, and 202 are not phosphorylated, AT180 (Pierce Perbio) recognizes phosphorylated Thr-231, and anti-Tau (pT^{212}) (BIOSOURCE) recognizes Thr(P)-212. Phosphorylation-independent antibodies M19G and anti-Tau-carboxyl-terminal (Tau-C-ter) are well characterized antisera, directed against the first 19 amino acids and the last 15 amino acids of Tau sequence, respectively (1, 2, 19, 20).

The anti-β-actin antibody was obtained from Sigma, anti-synaptophysin and anti-Pin1 were from Santa-Cruz (H-123), anti-neuronal nuclei was from Chemicon (MAB377), anti-cyclin D was from Upstate Biotechnology, and anti-phospho-Pin1 (Ser-16) was from Cell Signaling.

Electrophoresis and Immunoblotting—Equal volumes of cells harvested in Nu-Page sample buffer (Invitrogen) were denaturated at 100 °C during 5 min, loaded on 4–12% NuPAGE Novex gels (Invitrogen), and transferred to nitrocellulose. Membranes were blocked in Tris-buffered saline, pH 8, 0.05% Tween 20 with 5% skim milk or bovine serum albumin, and incubated with primary antibody. Membranes were incubated with horseradish peroxidase-labeled secondary antibody (goat anti-rabbit or anti-mouse IgGs, Sigma), and bands were visualized by chemiluminescence (ECL, Amersham Biosciences). Western blots were quantified by densitometry as described previously. Phosphorylated and total Tau (detected by M19G antibody) levels were normalized to β-actin (used as an internal loading control) for each blot (2). We previously verified that actin was not cleaved under the different treatments. Phosphorylated Tau levels were further normalized to total Tau to measure the exact phosphorylation state.

Immunocytochemistry—Cell cultures were fixed in cold 2% paraformaldehyde, 0.05% glutaraldehyde, 1 mM $MgCl_2$, 1 mM EGTA, and 30% glycerol in 70 mM PIPES, pH 6.8, for 1 h at room temperature. Permeabilization was carried out in 0.2% Triton X-100 in phosphate-buffered saline for 2 min. Cells were treated with $NaBH_4$ (10 mg/ml) for 7 min. After a 30-min sat-

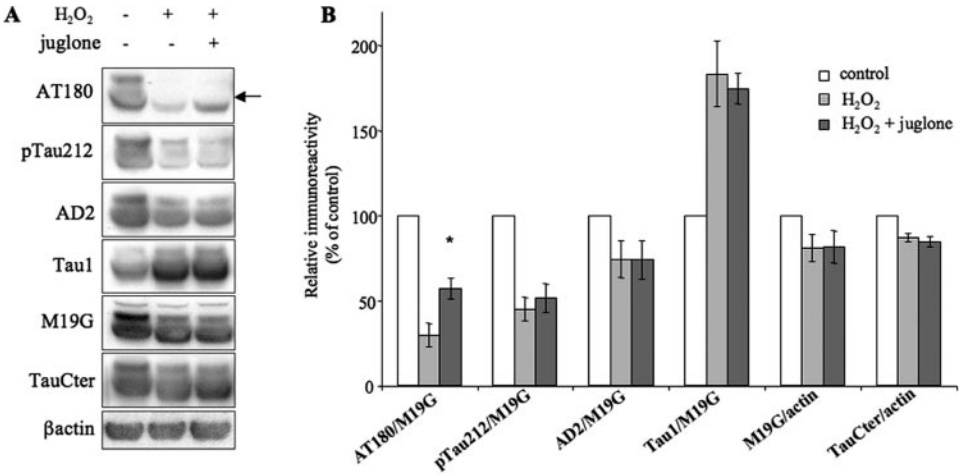

FIGURE 1. **Effect of juglone on H_2O_2-induced dephosphorylation of Tau.** *A*, Western blots analysis using phospho-dependent (AT180, anti-pTau212, AD2, Tau1) and phospho-independent (M19G, anti-TauCter) Tau antibodies. Cortical cultures were treated with 1 mM H_2O_2 for 1 h in the absence or presence of 1 μM juglone as described under "Experimental Procedures." For all Tau phosphorylation epitopes examined, H_2O_2 rapidly decreased phosphorylated Tau. Juglone pretreatment prevented H_2O_2-induced Tau dephosphorylation only at the epitope Thr-231 (*AT180*). *Arrow* indicates the 50-kDa molecular mass marker. *B*, densitometric analysis of Western blots using anti-total Tau and anti-phospho-Tau. Results are expressed as percentage of control. Data shown are the mean ± S.E. of three different experiments. *, $p < 0.001$ *versus* H_2O_2-treated cells.

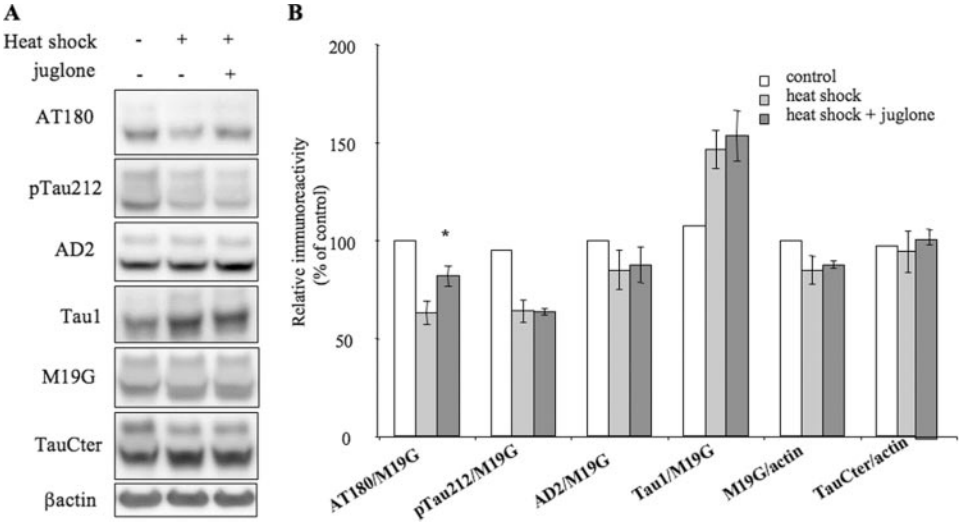

FIGURE 2. **Effect of juglone on heat shock-induced dephosphorylation of Tau.** *A*, Western blots analysis using phospho-dependent (AT180, anti-pTau212, AD2, Tau1) and phospho-independent (M19G, anti-TauCter) Tau antibodies. Cortical cultures were heated to 44 °C for 30 min in absence or presence of 1 μM juglone as described under "Experimental Procedures." Heat shock-induced dephosphorylation for all Tau epitopes was examined. Juglone pretreatment prevented heat shock-induced Tau dephosphorylation only at the epitope Thr231 (AT180). *B*, densitometric analysis of Western blots using anti-total Tau and anti-phospho-Tau. Results are expressed as percentage of control. Data shown are the mean ± S.E. of three different experiments. *, $p <$ 0.001 *versus* heat shock-treated cells.

uration in 4% bovine serum albumin, double immunostainings were carried out using anti-Pin1 and AT180 antibodies. Pin1 staining was revealed with a goat anti-rabbit IgG (H+L) antibody coupled to Alexa Fluor®488 and AT180 with a goat anti-mouse IgG (H+L) antibody coupled to Alexa Fluor®568 (Molecular Probes). Slides were analyzed by a Leica TCS NT confocal laser scanning microscope.

Analysis and Statistics—Results were expressed as means ± S.E. of at least three independent experiments. Statistics were performed by one-way analysis of variance followed by post-hoc Newman-Keuls.

RESULTS

Juglone Partially Reverses Oxidative Stress-induced Dephosphorylation of Thr(P)-231 Tau—As Tau is extensively phosphorylated in fetal neurons, primary neuronal cultures provide an appropriate model to study mechanisms regulating Tau dephosphorylation. H_2O_2 is a highly toxic product of oxidative stress mainly because of its free diffusion through the cells. Oxidative stress induced by hydrogen peroxide has been previously reported to induce hypophosphorylation of Tau through PP2A (12, 13, 21). Cortical cultures were exposed to 1 mM H_2O_2 for 1 h. Western blots (Fig. 1*A*) revealed with the anti-phosphorylated Tau antibodies AT180, anti-Tau (pT^{212}), and AD2 showed that H_2O_2 induced a decrease in phosphorylated Tau at threonine 231, threonine 212, and serines 396 and 404 (using the numbering convention of the longest human isoform of Tau), respectively. H_2O_2-induced dephosphorylation was confirmed by immunoblotting with the Tau1 antibody, which recognizes Tau only when it is not phosphorylated on serine residues 195, 198, 199, and 202. Dephosphorylated Tau detected by Tau1 increased after treatment with H_2O_2. Furthermore, H_2O_2 cell exposure induced a change of electrophoretic mobility and a decrease of total Tau detected by M19G antibody (20 ± 7%) and anti-Tau-C-ter (15 ± 3%), reflecting a global dephosphorylation and a partial degradation of Tau.

To assess the involvement of Pin1 in stress-induced dephosphorylation of Tau, juglone was used to inhibit its activity (15, 22). Neuronal cultures were preincubated for 2 h with 1 μM juglone prior H_2O_2 addition (Fig. 1*A*). We checked that 1 μM juglone did not alter either cell viability as assessed by MTS assay or the basal phosphorylation state of the various Tau epitopes tested (not shown).

Juglone pretreatment before H_2O_2 addition did not modify the levels of phosphorylated Tau epitopes Thr(P)-212-Tau, AD2, and Tau1. Conversely, inhibition of Pin1 by juglone partially prevented dephosphorylation of Tau at threonine 231 (27 ± 6%) as seen on Western blot revealed with the phospho-dependent Tau antibody AT180. Furthermore, as we reported above, H_2O_2 addition induced a partial decrease in total Tau as previously described (23, 24). Under the same treatments, no

significant changes were observed by immunoblotting for the neuron-specific marker NeuN and for the synaptic vesicle component synaptophysin indicating that there is no loss of cellular integrity (data not shown). These data suggest that Pin1 participates to H_2O_2-induced Tau dephosphorylation at threonine 231 under conditions that dephosphorylate a large set of phospho-Tau epitopes.

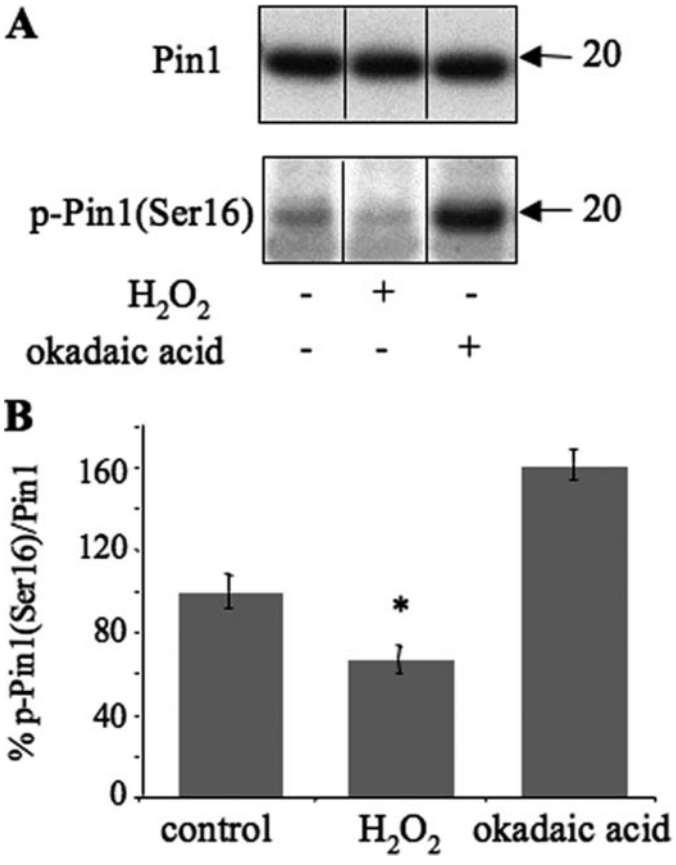

FIGURE 3. **Pin1 phosphorylation at serine 16.** *A*, Western blots using anti-total Pin1 and anti-Ser(P)-16-Pin1. Cortical cultures were treated with 1 mM H_2O_2 for 1 h. A low basal level of Ser(P)-16-Pin1 was observed. H_2O_2 treatment did not modify total Pin1 level but induced a decrease of phosphorylation on serine 16. 50 nM okadaic acid was used to increase basal phosphorylation. *B*, densitometric analysis of Western blots using anti-total Pin1 and anti-Ser(P)-16-Pin1. Results are expressed as percentage of control. Data shown are the mean ± S.E. of three different experiments. *, $p < 0.001$ *versus* control cells.

To extend the analysis of Pin1 involvement in stress mechanisms with PP2A activation, the juglone effect was tested on heat shock-induced Tau dephosphorylation. Previous studies described how heat shock caused rapid dephosphorylation of Tau in rats (14) and in cultured oligodendrocytes (13). In cortical cultures, heat shock (30 min at 44 °C) partially dephosphorylated Tau at serines 195, 198, 199, 202, 396, 404, and threonine 212 and 231. Juglone, like in H_2O_2 treatment, was only able to partially and selectively prevent heat shock-induced Tau dephosphorylation at Thr-231 (Fig. 2). These results suggest that heat shock-induced Tau dephosphorylation involves Pin1 at threonine 231. Thus, Pin1 participation to Tau dephosphorylation may be a common mechanism to various stresses.

Modulation of Pin1 Activation State through Its Phosphorylation on Serine 16—Because the direct rotamase activity of Pin1 could only be visualized directly by NMR spectroscopy, we first analyzed phosphorylation on serine 16, which is located inside the WW domain, and recently reported to inhibit Pin1 binding to its substrates (25). Ser(P)-16 was analyzed in basal conditions and under H_2O_2 and/or okadaic acid treatment (at a 50 nM concentration specifically inhibiting PP2A) (Fig. 3).

Total Pin1 levels were not modified under H_2O_2 and/or okadaic acid treatments. Phosphoserine-16-Pin1 antibody only slightly detected Pin1 protein in control conditions indicating that Pin1 could bind to its substrates, in our culture conditions. H_2O_2 further decreased Pin1 Ser-16 phosphorylation, suggesting that Pin1 has a better ability to bind its substrates in stress-induced conditions. Conversely, the phosphatase inhibitor okadaic acid induced a strong phosphorylation of Pin1 suggesting that dephosphorylation of phosphoserine 16 would be mediated, at least partly, by PP2A (Fig. 3).

Oxidative Stress-induced Dephosphorylation of Tau in Pin1-deficient Cultures—To challenge our results obtained with juglone inhibition, we therefore tested whether Pin1 deficiency would modify the response to the most efficient stress-induced Tau dephosphorylation, H_2O_2. We compared the effect of H_2O_2 on AT180, Thr(P)-212-Tau, AD2, Tau1, M19G, and anti-Tau-C-ter epitopes in Pin1 and Pin1-deficient cortical neuronal cultures. We first verified by genotyping and immunoblotting the absence of Pin1 in cells cultured from knock-out Pin1 embryos (Fig. 4). Regarding the effects of H_2O_2 on Tau dephosphorylation, data were similar in rat and mice primary neuronal cell cultures (Fig. 1 *versus* Fig. 5, *panels wt*). H_2O_2-induced dephosphorylation of Tau revealed with anti-Thr(P)-212-Tau, AD2, Tau 1, M19G, and anti-Tau-C-ter remained unchanged in Pin1-deficient neuronal cultures as compared with wild type cultures. Interestingly, significant protection to H_2O_2 treatment in Pin1-deficient neuronal cultures was observed only using the anti-phosphorylated Tau antibody

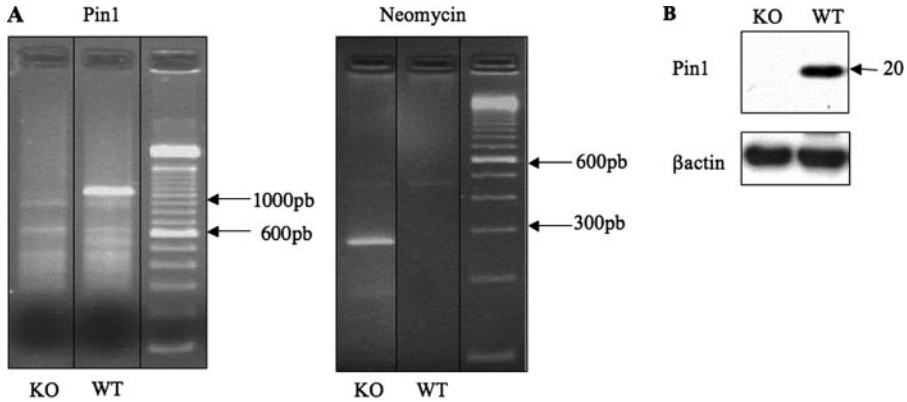

FIGURE 4. **Detection of Pin1 in neuronal cultures from wild type and Pin1-deficient embryos.** *A*, representative gel analysis of the PCR product of the two genotypes of embryos. *B*, Pin1 was detected by Western blots in cultures from wild type or Pin1-deficient embryos.

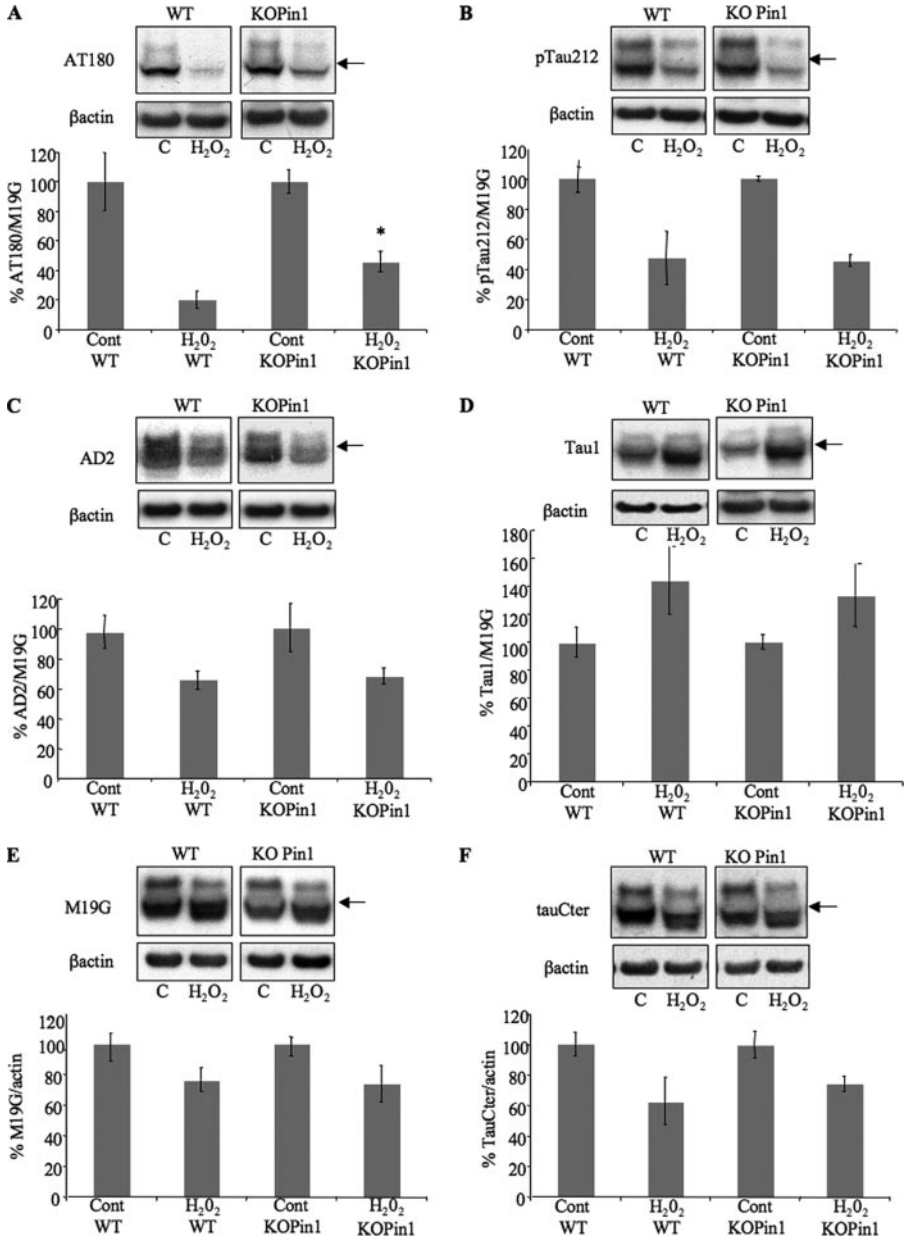

FIGURE 5. **Pin1 deficiency prevents H_2O_2-induced dephosphorylation of Tau at Thr-231 site.** Western blots analysis using phospho-dependent (*A*, AT180; *B*, anti-pTau212; *C*, AD2; *D*, Tau1) and phospho-independent (*E*, M19G; *F*, anti-TauCter) Tau antibodies. Cortical cultures were prepared from individual wild type (*WT*) or Pin1-deficient embryos and treated with 1 mM H_2O_2. Tau dephosphorylation was significantly reduced at epitope Thr-231 (*A*, AT180) in H_2O_2-treated Pin1-deficient cultures compared with wild type. At the opposite, no difference was observed for the other phospho-Tau epitopes tested and for total Tau. The densitometric analysis was represented for each epitope tested. Each data point represents combined values from four separate embryos. *, $p < 0.01$ *versus* H_2O_2-treated wild type cells. *Arrows* indicate the 50-kDa molecular mass marker.

AT180 (Fig. 5). This provides further evidence that Pin1 is specifically involved in Thr(P)-231 Tau dephosphorylation.

Thr(P)-231 Tau Distribution in Wild Type or Pin1-deficient Cortical Neurons—Confocal microscopy using anti-Pin1 antibody revealed that Pin1 localizes both to the nucleus and the cellular body in cortical neurons (Fig. 6). A strong presence of Pin1 was also observed in neurites and varicosities. On parallel, AT180 mainly stained axons and cellular body. A double staining with both antibodies (merge) revealed partial colocalization between anti-Pin1 and AT180 antibodies to cellular bodies and neurites. These data correlate a possible Pin1 and Thr(P)-231 Tau colocalization, facilitating the access of Pin1 to Tau and specific isomerization of the Thr(P)-231-proline peptide bond under dephosphorylation conditions. In Pin1-deficient neurons, a similar AT180 immunoreactivity was found (Fig. 6, *bottom panel*).

Rescue of H_2O_2-induced Dephosphorylation of Tau in Pin1-deficient Neurons—To learn the precise role of the Pin1 prolyl isomerase activity in H_2O_2-induced Tau dephosphorylation, Pin1-deficient cultures were transfected at 7 days *in vitro* with cDNA coding for wild type Pin1 or a catalytic mutant where Ser-115 was replaced by a glutamic acid, S115E. Three days after transfection, we observed by Western blotting a strong expression of both wild type and Glu-115-mutated Pin1 as detected with anti-Pin1 antibody (Fig. 7*A*). These results proved the high efficiency of transfection of the modified Ca^{2+}-phosphate method used. Thus, the effect of wild type Pin1 and Glu-115 mutant overexpression on H_2O_2-induced dephosphorylation of Tau in Pin1-deficient neurons was tested by immunoblotting.

First, to test the efficiency of wild type Pin1 and catalytic mutant Glu-115 activities, the effect of their overexpression on cyclin D levels were analyzed in COS cells and Pin1-deficient cortical cultures (Fig. 7*A*). To assess the efficiency of both constructs, the amounts of cyclin D, which was shown as stabilized at both mRNA and protein levels by Pin1, was quantified by immunoblotting (26–28). A cyclin D increase was observed in both cultures overexpressing Pin1. Conversely, such cyclin D1 increase was not visualized with the Glu-115 mutant overexpression (Fig. 7*B*).

Three days after transfection, Pin1-deficient cultures were treated by H_2O_2 for 30 min to mildly dephosphorylate Tau. We compared H_2O_2 treatment effect on AT180, Thr(P)-212-Tau, AD2, Tau1, and M19G epitopes in Pin1-deficient cortical neuronal culture transfected with no coding cDNA, wild type, or Glu-115 mutated-Pin1 (Fig. 8*A*). No effect of wild type or Glu-115-mutated Pin1 overexpression was observed for the phosphorylated Tau epitopes Thr(P)-212-Tau, AD2, and Tau1. However, wild type Pin1 overexpression increased Tau dephos-

phorylation only using the anti-phosphorylated Tau antibody AT180. The catalytic Glu-115 mutant was not as effective because its overexpression partially prevented the increased dephosphorylation of Tau (Fig. 8*B*).

H_2O_2-induced dephosphorylation of Tau at the Thr-231 site in transfected Pin1-deficient cultures was illustrated by immunofluorescence studies and analyzed by confocal microscopy (Fig. 9). Overexpression of either wild type Pin1 or the Glu-115 mutant induced a strong staining detected by anti-Pin1 antibody in transfected cells. In cells exhibiting a clear neuronal morphology, an AT180 immunoreactivity was observed. Then, we looked for cells with similar morphology that exhibited Pin1 immunoreactivity. A weak AT180 staining was found in cells transfected with Pin1, whereas AT180 immunoreactivity in cells transfected with the Pin1 mutant Glu-115 was similar to the one observed in non-transfected cells. Altogether, these observations further strengthen the role of the catalytic domain of Pin1 prolyl isomerase in the H_2O_2-induced dephosphorylation of Tau at Thr-231 site.

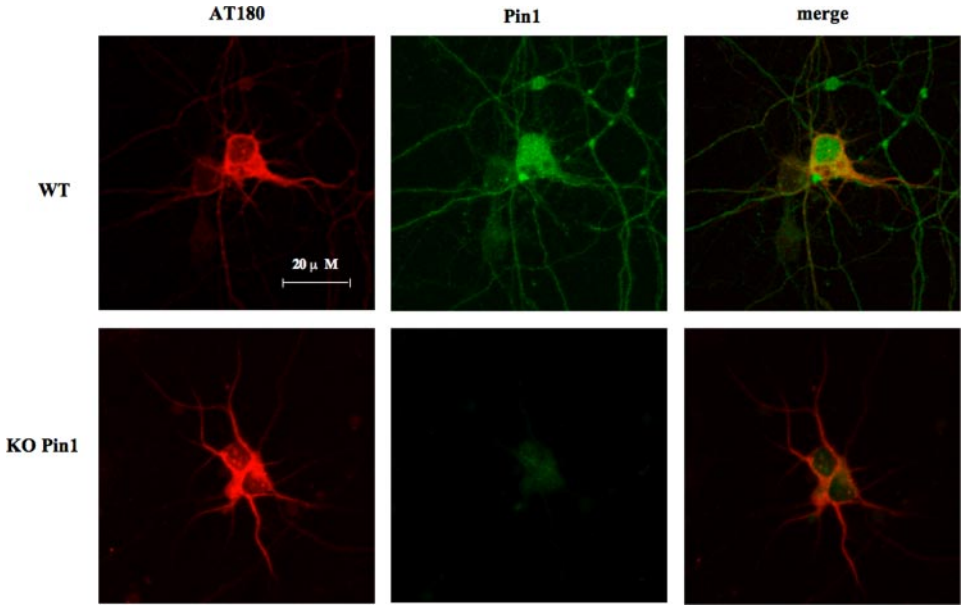

FIGURE 6. **Thr(P)-231-Tau localization in wild type and Pin1-deficient cortical cultures.** Double immunostaining of wild type (*WT*) and Pin1-deficient cortical cultures for Thr(P)-231-Tau (AT180) and total Pin1. Thr(P)-231 was localized to cell bodies and axons. Pin1 was found strongly in nucleus but also in cellular bodies and neurites. Absence of Pin1 did not alter Thr(P)-231-Tau localization in Pin1-deficient cells. *KO*, knock-out.

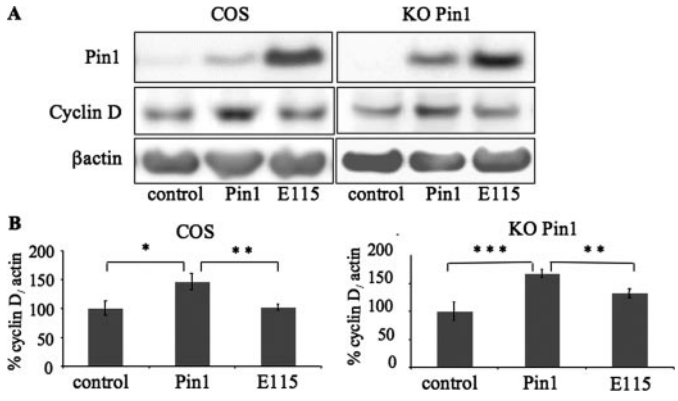

FIGURE 7. **Characterization of Pin1 and Glu-115 (*E115*) mutant overexpression in COS cells and Pin1-deficient cultures.** *A*, Western blots analysis using anti-total Pin1 and anti-cyclin D antibodies of COS cells and Pin1-deficient cortical cultures transfected with cDNA coding for Pin1 or the catalytic Pin1 mutant Glu-115. Pin1 and Glu-115 mutant were strongly expressed in both cell types. Pin1 overexpression induced a cyclin D increase in COS cells and Pin1-deficient cultures. Overexpression of Glu-115 mutant fully prevented cyclin D increase in COS cells and partially in Pin1-deficient cultures. *KO*, knock-out. *B*, densitometric analysis of Western blots using anti-total Pin1 and anti-cyclin D antibodies. Results are expressed as percentage of control. Data shown are the mean ± S.E. of three different experiments. *, $p < 0.01$ Pin1 *versus* control cells; **, $p < 0.05$ Glu-115 *versus* Pin1; ***, $p = 0.001$ Pin1 *versus* control cells.

DISCUSSION

Tau phosphorylation is likely to be a main actor in neurofibrillary degeneration. However, the deregulation of Tau phosphorylation is still a key question in AD pathogenesis. Protein phosphorylation is regulated through a balance of kinases and phosphatases. Recently, glycogen synthase kinase-3β (GSK3β) inhibitors were shown to slow down neurofibrillary degeneration in a Tau transgenic model, suggesting that GSK3β is a main kinase involved in Tau phosphorylation (29). Conversely, recent data also indicated that the lack of phosphatase activity in AD brains might lead to Tau hyperphosphorylation (30). Finally, a new regulator of phosphorylation was also involved, the peptidylprolyl *cis/trans*-isomerase Pin1. Many phosphorylation sites defined as pathological are phospho-Ser/Thr followed by Pro. The conformation of this peptide bond is crucial for the action of phosphatases. PP2A is only able to dephosphorylate Ser(P)/Thr(P)-Pro when it is in a trans conformation but not in a cis conformation (11). The interest in Pin1 as a key actor in neurofibrillary degeneration is supported by the fact that Pin1 knock-out mice develop neurofibrillary tangles (7, 8).

Pin1 was reported to present a high affinity for both the Thr(P)-231 and Thr(P)-212 of Tau protein (7, 9, 10). However, whether binding of Pin1 to Tau leads to isomerization of a single site or of multiple Ser(P)/Thr(P)-Pro sites *in vivo* is still unknown (31). Moreover, the enzyme activity of Pin1 is dependent on the level of phosphorylation of its substrate. For instance, Tau peptide with a unique phosphorylation site (Thr(P)-212) is a better substrate than multiphosphorylated peptides. Indeed, the WW domain of Pin1 may act as a negative regulator of the cis/trans isomerization activity when Pin1 substrates are multiphosphorylated (31). Thus, it is necessary to determine the effect of Pin1 *in vivo* on phosphorylated full-length Tau protein. Six Ser/Thr-Pro residues (Ser-199, Ser-202, Thr-212, Thr-231, Ser-396, and Ser-404) dephosphorylated by PP2A were studied to cover the entire Tau molecule (32).

Finally, further demonstration is needed to highlight Pin1

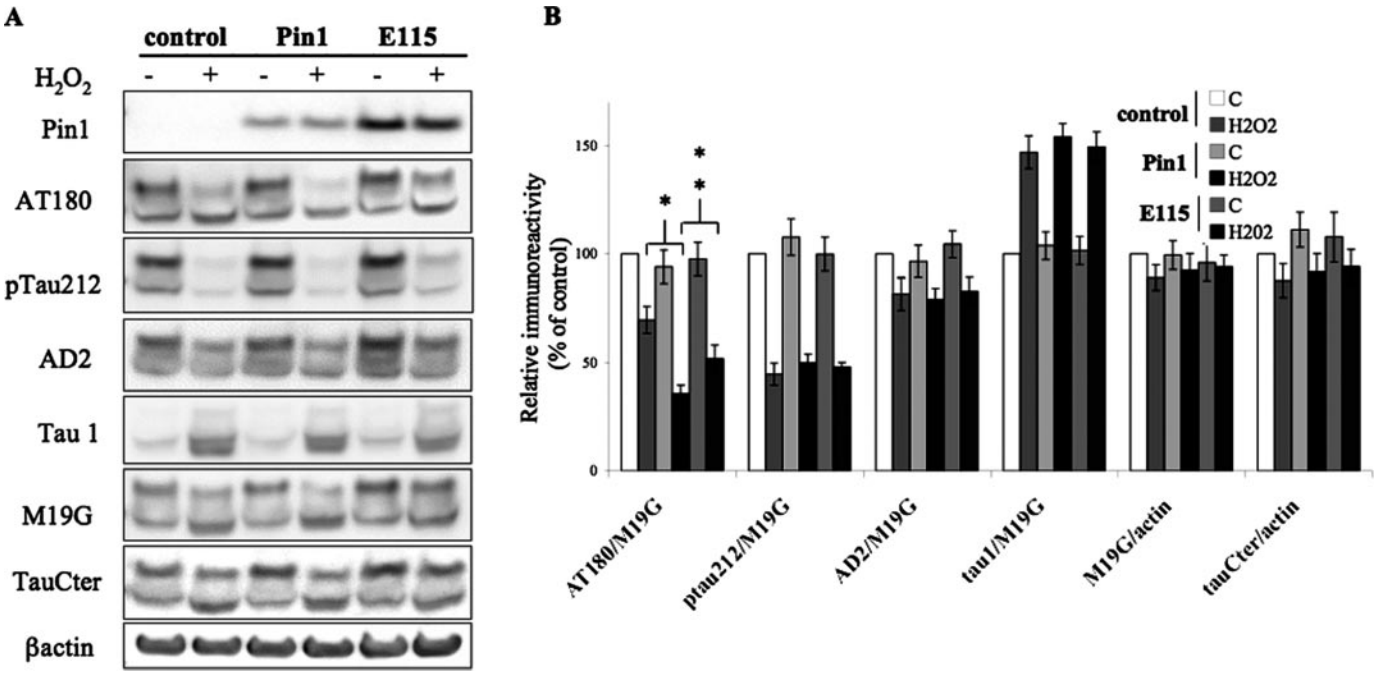

FIGURE 8. **Pin1 overexpression increases H_2O_2-induced dephosphorylation of Tau at the Thr-231 site in transfected Pin1-deficient cultures.** *A*, Western blots analysis using phospho-dependent (AT180, anti-pTau212, AD2, Tau1) and phospho-independent (M19G, anti-TauCter) Tau antibodies of Pin1-deficient cortical cultures transfected with cDNA coding for Pin1 or the catalytic Pin1 mutant Glu-115 (*E115*). Cortical cultures were treated with 1 mM H_2O_2 for 30 min at 37 °C. Pin1 overexpression increased H_2O_2-induced Tau dephosphorylation only at epitope Thr-231 (*AT180*). Mutation of the catalytic site partially prevented Pin1 effect on H_2O_2-induced Thr(P)-231 dephosphorylation. *B*, densitometric analysis of Western blots using anti-total Tau and anti-phospho-Tau. Results are expressed as percentage of control. Data shown are the mean ± S.E. of three different experiments. *, $p < 0.001$ H_2O_2-treated Pin1 overexpressing cells *versus* H_2O_2-treated control cells; **, $p = 0.001$ H_2O_2-treated Glu-115 *versus* H_2O_2-treated Pin1 overexpressing cells.

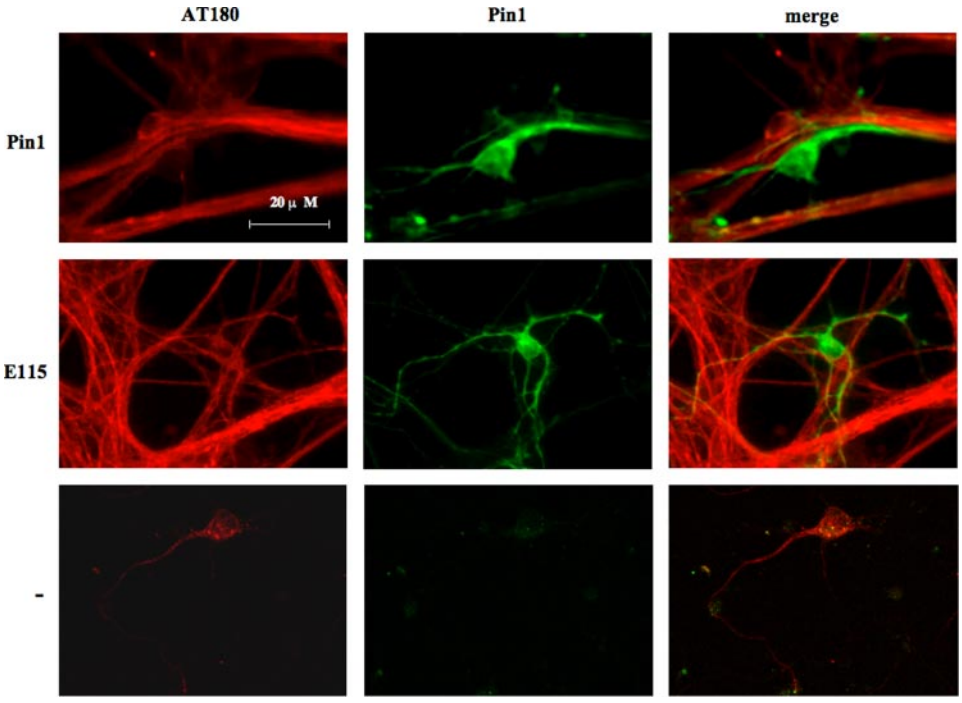

FIGURE 9. **Modulation of Thr(P)-231 in transfected Pin1-deficient neurons.** Double immunostaining of Pin1-deficient neurons overexpressing Pin1 or the catalytic mutant Glu-115 (*E115*) for Thr(P)-231 Tau (AT180) and total Pin1. Pin1 but not Glu-115 overexpression strongly decreases Thr(P)-231 staining. −, non-transfected Pin1-deficient neuronal cell culture.

and Tau relationships in dephosphorylation mechanisms. Two stresses leading to PP2A activation were used to assess the involvement of Pin1 in Tau dephosphorylation.

First, the present data indicate that Pin1 preferentially modulates stress-induced dephosphorylation of Tau on Thr-231. Juglone, a pharmacological Pin1 inhibitor was used to inactivate rotamase activity. Pin1 inhibition led to a decreased effect of both oxidative and heat stresses induced dephosphorylation of Thr(P)-231 Tau without affecting dephosphorylation of other sites. Nevertheless, juglone inhibits numerous parvulins as well as other enzymes comprising Cys residues in their catalytic domains (15, 22, 33–35). Other pharmacological Pin1 inhibitors are available (36–39), but Pin1-deficient cells are likely to be the most useful tools. We have set up an original approach by developing primary neuronal cultures from Pin1 knock-out mice.

Thus, the pharmacological effect of juglone observed in Tau dephosphorylation experiments in wild type neurons was then compared with similar work in Pin1 knock-out neurons. Pin1 deficiency led to a decreased effect of H_2O_2-induced dephosphorylation of Thr(P)-231 Tau without affecting dephosphorylation of other sites. It further supports that lack of Pin1 activity leads to a deficient Tau dephosphorylation at Thr(P)-231 under stress conditions. Never-

theless, we cannot speak about an exclusive selectivity of Pin1 isomerase activity for Thr(P)-231 because only 6 of the 17 Ser(P)/Thr(P)-Pro sites present on Tau were investigated. However, our results allow for demonstrating that Pin1 does not modulate H_2O_2-induced dephosphorylation of Thr(P)-212 Tau despite recent NMR results showing an even higher affinity of Pin1 for Thr(P)-212 than for Thr(P)-231 Tau (10). Anyhow, this does not preclude that Pin1 may not isomerize Thr(P)-212 Tau in cells under different dephosphorylating conditions or in specific types of neurons or brain area.

Neither juglone nor Pin1 deficiency completely abolished the H_2O_2-induced effect, indicating that Thr(P)-231 may be dephosphorylated without Pin1. It is not surprising because only a small percentage of the peptide bound is in a cis conformation. Most of it is already in a trans conformation. This could be because of a direct, although slower, isomerization of Thr(P)-231-Pro peptide bond, or possibly another isomerase enzyme.

Pin1 is constituted of two structural domains, a C-terminal catalytic and an anchoring N-terminal WW domain. Phosphorylation of the WW domain on Ser-16 abolishes its capacity to interact with phosphoepitopes (25). Our results indicate that in control conditions, a majority of Pin1 is in a favorable state to bind to its substrates. Stress further increases Pin1 ability to bind Tau through dephosphorylation of Ser(P)-16 WW domain of Pin1. So, stress-induced dephosphorylation of Ser(P)-16-Pin1 increases its potential to bind Tau and isomerase Thr(P)-231 site. The transfection of either functional Pin1 or a catalytic defective mutant in Pin1-deficient primary neuronal cultures further supports the role of the rotamase activity in the stress-induced Tau dephosphorylation process.

In differentiated cultured neurons, Pin1 was localized not only in the nucleus but also in the cytoplasm and the neurite network (40). Histochemical data previously reported cytoplasmic and nuclear Pin1 in the brain (8). On one hand, the partial co-localization of Pin1 with Thr(P)-231-Tau illustrates their close vicinity in the neuron, making possible a rapid interaction between both proteins. On the other hand, Thr(P)-231 Tau localization is likely to be Pin1-independent, as no change was observed in Pin1-deficient neurons.

As Thr-231 is a crucial site for the microtubule binding activity of Tau, in the brain, reduction or absence of Pin1 isomerase activity would be able to significantly modify the response of Tau to pathological assault. As oxidative stress is involved early in AD, Pin1 alteration might be a very preliminary request to initiate neurofibrillary tangles and, consequently, restoration of Pin1 activity may represent an attractive target for future therapeutics drugs.

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REFERENCES

1. Buée, L., Bussière, T., Buée-Scherrer, V., Delacourte, A., and Hof, P. R. (2000) *Brain Res. Rev.* **33,** 95–130
2. Hamdane, M., Sambo, A. V., Delobel, P., Bégard, S., Violleau, A., Delacourte, A., Bertrand, P., Benavides, J., and Buée, L. (2003) *J. Biol. Chem.* **278,** 34026–34034
3. Cho, J. H., and Johnson, G. V. W. (2003) *J. Biol. Chem.* **278,** 187–193
4. Cho, J. H., and Johnson G. V. W. (2004) *J. Neurochem.* **88,** 349–358
5. Hoffmann, R., Lee, V. M., Leight, S., Varga, I., and Otvos, L., Jr. (1997) *Biochemistry* **36,** 8114–8124
6. Jicha, G. A., Lane, E., Vincent, I., and Otvos, L. (1997) *J. Neurochem.* **69,** 2087–2095
7. Lu, P. J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) *Nature* **399,** 784–788
8. Liou, Y. C., Sun, A., Ryo, A., Zhou, X. Z., Yu, Z. X., Huang, H. K., Uchida, T., Bronson, R., Bing, G., Li, X., Hunter, T., and Lu, H. P. (2003) *Nature* **424,** 556–561
9. Wintjens, R., Wieruszeski, J. M., Drobecq, H., Rousselot-Pailley, P., Buee, L., Lippens, G., and Landrieu, I. (2001) *J. Biol. Chem.* **276,** 25150–25156
10. Smet, C., Sambo, A. V., Wieruszeski, J. M., Leroy, A., Landrieu, I., Buee, L., and Lippens, G. (2004) *Biochemistry* **43,** 2032–2040
11. Zhou, X. Z., Kops, O., Werner, A., Lu, P. J., Shen, M., Stoller, G., Kullertz, G., Stark, M., Fischer, G., and Lu, K. P. (2000) *Mol. Cell.* **6,** 873–883
12. Davis, D. R., Anderton, B. H., Brion, J. P., Reynolds, C. H., and Hanger, D. P. (1997) *J. Neurochem.* **68,** 1590–1597
13. Goldbaum, O., and Richter-Landsberg, C. (2002) *Glia* **40,** 271–282
14. Papasozomenos, S. C., and Papasozomenos, T. (1999) *J. Alzheimer Dis.* **1,** 147–153
15. Chao, S. H., Greenleaf, A. L., and Price, D. H. (2001) *Nucleic Acids Res.* **29,** 767–773
16. Fujimori, F., Takahashi, K., Uchida, C., and Uchida, T. (1999) *Biochem. Biophys. Res. Commun.* **265,** 658–663
17. Ranganathan, R., Lu, K. P., Hunter, T., and Noel, J. P. (1997) *Cell* **89,** 875–886
18. Jiang, M., Deng, L., and Chen, G. (2004) *Gene Ther.* **11,** 1303–1311
19. Mailliot, C., Sergeant, N., Bussiere, T., Caillet-Boudin, M. L., Delacourte, A., and Buée, L. (1998) *FEBS Lett.* **433,** 201–204
20. Delobel, P., Flament, S., Hamdane, M., Mailliot, C., Sambo, A. V., Bégard, S., Sergeant, N., Delacourte, A., Vilain, J. P., and Buée, L. (2002) *J. Neurochem.* **83,** 412–420
21. Zambrano, C. A., Egana, J. T., Nunez, M. T., Maccioni, R. B., and Gonzales-Billault, C. (2004) *Free Radic. Biol. Med.* **36,** 1393–1402
22. Hennig, L., Christner, C., Kipping, M., Schelbert, B., Rucknagel, K. P., Grabley, S., Kullertz, G., and Fisher, G. (1998) *Biochemistry* **37,** 5953–5960
23. Rametti, A., Esclaire, F., Yardin, C., and Terro, F. (2004) *J. Biol. Chem.* **279,** 54518–54528
24. Canu, N., Dus, L., Barbato, C., Ciotti, M. T., Brancolini, C., Rinaldi, A. M., Novak, M., Cattaneo, A., Bradbury, A., and Calissano, P. (1998) *J. Neurosci.* **18,** 7061–7074
25. Lu, P. J., Zhou, X. Z., Liou, Y. C., Noel, J. P., and Lu, K. P. (2002) *J. Biol. Chem.* **277,** 2381–2384
26. Wulf, G. M., Ryo, A., Wulf, G. G., Lee, S. W., Niu, T., Petkova, V., and Lu, K. P. (2001) *EMBO J.* **20,** 3459–3472
27. Liou, Y. C., Ryo, A., Huang, H. K., Lu, P. J., Bronson, R., Fujimori, F., Uchida, T., Hunter, T., and Lu, K. P. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 1335–1340
28. Hamdane, M., Smet, C., Sambo, A. V., Leroy, A., Wieruszeski, J. M., Delobel, P., Maurage, C. A., Ghestem, A., Wintjens, R., Begard, S., Sergeant, N., Delacourte, A., Horvath, D., Landrieu, I., Lippens, G., and Buee, L. (2002) *J. Mol. Neurosci.* **19,** 275–287
29. Noble, W., Planel, E., Zehr, C., Olm, V., Meyerson, J., Suleman, F., Gaynor, K., Wang, L., LaFrancois, J., Feinstein, B., Burns, M., Krishnamurthy, P., Wen, Y., Bhat, R., Lewis, J., Dickson, D., and Duff, K. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 6990–6995
30. Tanimukai, H., Grundke-Iqbal, I., and Iqbal, K. (2005) *Am. J. Pathol.* **6,** 1761–1771
31. Smet, C., Wieruszeski, J. M., Buée, L., Landrieu, I., and Lippens, G. (2005) *FEBS Lett.* **579,** 4159–4164
32. Liu, F., Grundke-Iqbal, I., Iqbal, K., and Gong, G. X. (2005) *Eur. J. Neurosci.* **22,** 1942–1950
33. Vos, R. M., Van Ommen, B., Hoekstein, M. S., De Goede, J. H., and Van Bladeren, P. J. (1989) *Chem. Biol. Interact.* **71,** 381–392

34. de Castro, E., Hegi de Castro, S., and Johnson, T. E. (2004) *Free Radic. Biol. Med.* **37,** 139–145
35. Paulsen, M. T., and Ljungman, M. (2005) *Toxicol. Appl. Pharmacol.* **209,** 1–9
36. Zhang, Y., Fussel, S., Reimer, U., Schutkowski, M., and Fischer, G. (2002) *Biochemistry* **41,** 11868–11877
37. Uchida, T., Takamiya, M., Takahashi, M., Miyashita, H., Ikeda, H., Terada, T., Matsuo, Y., Shirouzu, M., Yokoyama, S., Fujimori, F., and Hunter, T. (2003) *Chem. Biol.* **10,** 15–24
38. Bayer, E., Thutewohl, M., Christner, C., Tradler, T., Osterkamp, F., Waldmann, H., and Bayer, P. (2005) *Chem. Commun.* (*Camb.*) **4,** 516–518
39. Smet, C., Duckert, J. F., Wieruszeski, J. M., Landrieu, I., Buee, L., Lippens, G., and Deprez, B. (2005) *J. Med. Chem.* **48,** 4815–4823
40. Hamdane, M., Dourlen, P., Bretteville, A., Sambo, A. V., Ferreira, S., Ando, K., Kerdraon, O., Bégard, S., Geay, L., Lippens, G., Sergeant, N., Delacourte, A., Maurage, C. A., Galas, M. C., and Buée, L. (January 17, 2006) *Mol. Cell. Neurosci.*, 10.1016/j.mcn.2006.03.006

The Peptidylprolyl *cis/trans*-Isomerase Pin1 Modulates Stress-induced Dephosphorylation of Tau in Neurons: IMPLICATION IN A PATHOLOGICAL MECHANISM RELATED TO ALZHEIMER DISEASE

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