The Peptidyl Prolyl *cis*/*trans*-Isomerase Pin1 Recognizes the Phospho-Thr212-Pro213 Site on Tau†

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ABSTRACT: The interaction between the neuronal Tau protein and the Pin1 prolyl *cis*/*trans*-isomerase is dependent on the phosphorylation state of the former. The interaction site was mapped to the unique phospho-Thr231-Pro232 motif, despite the presence of many other Thr/Ser-Pro phosphorylation sites in Tau and structural evidence that the interaction site does not significantly extend beyond those very two residues. We demonstrate here by NMR and fluorescence mapping that the Alzheimer's disease specific epitope centered around the phospho-Thr212-Pro213 motif is also an interaction site, and that the sole phospho-Thr-Pro motif is already sufficient for interaction. Because a detectable fraction of the Pro213 amide bond in the peptide centered around the phospho-Thr212-Pro213 motif is in the *cis* conformation, catalysis of the isomerization by the catalytic domain of Pin1 could be investigated via NMR spectroscopy.

Tau is a microtubule-associated protein that occurs mainly in neurons (*1*). Although derived from a single gene, it can exist through alternative splicing as six isoforms that are developmentally regulated (*2*). Further variability is introduced by posttranslational phosphorylation, with a fetal isoform of Tau carrying significantly more phosphate groups in embryonic stages than the six Tau isoforms in adult brain (*3*). A decade of research has led to the discovery of a large number of kinases that can phosphorylate Tau on specific threonine (Thr) or serine (Ser) residues. Mapping of the phosphorylation sites was done with specific antibodies (*4*−*6*) or with protein chemistry techniques, involving mass spectroscopy and/or Edman degradation (*3*, *7*, *8*). It was found that many kinases belong to the proline (Pro)-directed protein kinase family, with an enzymatic activity for one of the 17 Ser/Thr-Pro motifs in full-length Tau. Other kinases, however, such as PKA[1] were found to phosphorylate Ser or Thr residues that are not followed by a Pro (*9*), whereas GSK-3β can modify both Ser and Thr residues that are or are not followed by a Pro (*10*−*12*).

The main motivation for this extensive research was and remains twofold. First, phosphorylation was found to modulate the binding of Tau to the microtubules, with a lower affinity for the phosphorylated Tau, and might as such influence the dynamic equilibrium of the tubulin/microtubule system (*13*). Second, in the paired helical filaments (PHF), one of the hallmarks of Alzheimer's disease of which Tau is the main component, the protein is heavily phosphorylated (*1*). Hyperphosphorylation in the later life stages might reflect an erroneous reactivation of the cell cycle in the aging neurons, proposed on the basis of the agreement between AD and mitosis-related phosphorylation patterns (*14*−*16*). Still, the relationship between phosphorylation and aggregation is not clear, and certain phosphorylation sites (Ser214 and Ser262) were recently described as being protective against aggregation (*17*).

The phospho-epitope mapping by immunochemistry heavily relies on a number of specific antibodies that recognize (i) the protein in its unphosphorylated soluble form, (ii) certain phospho-epitopes, or (iii) the phosphorylated protein aggregated into PHF tangles as found in the diseased brain. Examples of the latter are the AT100 and PHF-27/TG3 antibodies that recognize the AD specific phosphorylation sites composed of the Thr212−Ser214 and Thr231−Ser235 epitopes, respectively (*14*, *18*). Whereas those antibodies are commonly classified as being "conformation-dependent", the precise conformational changes that they detect have still not been fully elucidated (*19*, *20*). Because many of the phosphorylation sites are Pro-directed, one possible hypothesis is that the Pro conformation would change upon phosphorylation and/or aggregation. The Pro residue is indeed unique in the sense that the energetic barrier between its *trans* and *cis* conformation is significantly lower than for

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[1] Abbreviations: PKA, protein kinase A; GSK-3β, glycogen synthase kinase 3β; AD, Alzheimer's disease; PP2A, protein phosphatase 2A; PHF, paired helical filaments; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; DMF, dimethylformamide; RP-HPLC, reverse phase high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; IPTG, isopropyl thiogalactoside; PBS, phosphate-buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser exchange spectroscopy; EXSY, exchange spectroscopy; FRET, fluorescence resonance energy transfer; K_D, dissociation constant; TFE, trifluoroethanol.

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all other amino acids, leading to a higher population of the *cis* form. In small peptides, this population is typically on the order of a few percent, but in the context of a folded protein, Pro residues can be found that are completely in the *cis* conformation.

Indirect evidence of a structural role of the proline conformation comes from the recent finding that Pin1, a prolyl *cis*/*trans*-isomerase that is essential for the cell cycle and highly conserved within all eukaryotic species (*21*, *22*), interacts with Tau (*23*). Pin1 was reported to restore the microtubule binding capacity of Cdc2/cyclin B-phosphorylated Tau (*23*). This might result from a direct physical interaction between both partners, or alternatively, Pin1 might assist proline-directed phosphatase PP2A in its catalytic activity on phosphorylated Tau. Indeed, at least at the level of short peptide substrates, PP2A only catalyzes the dephosphorylation of a threonine when it is followed by a proline residue in the *trans* conformation (*24*). Finally, the large reservoir of phosphorylated Tau in the PHF tangles might lead to depletion of soluble Pin1, and as such interfere with Pin1's other functions. Because Pin1 is a key regulator of the mitotic transition, the earlier finding that Alzheimer's disease might be related to a reactivation of the cell cycle (*14*−*16*) is in agreement with a functional role for Pin1 in AD.

Pin1 is constituted by two domains (*25*) both essential for its *in vivo* activity: a WW binding domain (*26*) which binds specifically pThr/pSer-Pro motifs and a peptidyl-prolyl *cis*/*trans*-isomerase domain which can act catalytically to promote conformational changes in pThr/pSer−Pro bonds by accelerating the intrinsic slow *cis*/*trans* isomerization of these bonds at least on small peptidic substrates (*27*). When the interaction between phosphorylated Tau and Pin1 was reported, the authors identified a unique interaction site at the phosphorylated Thr231 residue. Combined peptide mapping and mutagenesis studies indicated that phosphorylation of this proline-directed site, which is phosphorylated significantly in both fetal and adult rat brains (*3*) but is also one of the phosphorylation sites detected on PHF Tau, is both necessary and sufficient for the interaction with Pin1. Thr231 can be phosphorylated by GSK-3β, but only after phosphorylation of Ser235 by CDK5 or MAPK (*6*, *28*, *29*). It can be directly phosphorylated by CDK5 in the presence of heparin (*30*), and with a low efficiency by the p35cdc2−cyclin A complex (*31*).

Structural details of the interaction of Pin1 with its various substrates come from several high-resolution X-ray and NMR structures. Pin1 in complex with peptide substrates derived from the C-terminal domain of the polymerase II, the CDC25 phosphatase, or Tau itself indicates a dominant role for the WW domain in the interaction, with an aromatic clamp formed by one of the Trp residues and a Tyr holding onto the Pro side chain, whereas Arg and Ser in the loop region connecting the two β-strands make hydrogen bonds with the phosphorylated side chain of the Thr (*32*, *33*). The interaction seems, however, mainly limited to this precise dipeptide, raising the question of the structural basis for the reported selectivity. In a recent NMR study, the only conformational selectivity we could detect was at the level of the proline isomerization state, with the pThr-Pro moiety interacting only when the Pro is in the *trans* conformation (*33*).

On the basis of the discrepancy between this structural information and the reported uniqueness of the Pin1 interaction site on Tau, we decided to further investigate this aspect by NMR spectroscopy. As the Thr212 residue is one of the few proline-directed and PHF specific phosphorylation sites, we centered our peptide on this residue. Another reason for the choice of this precise peptide is that the two reported interaction sites on human CDC25C, Thr47 and Thr68, are separated by exactly the same number of residues as Thr212 and Thr231 of Tau (*34*). Although the sequences of both proteins between their respective two threonine residues do not align, they both are considered random coil polymers without well-defined structure, and their separation might therefore be the relevant structural factor. Moreover, as other sites in the direct vicinity of the Thr212 site can equally be phosphorylated (Ser214 and Thr217), we have first investigated the influence of phosphorylation on the conformation of the peptide, by performing a detailed NMR analysis of the non-, mono-, di-, and triphosphorylated peptide. Their interaction with Pin1 was studied at the level of binding to the WW domain in the full-length protein, and the conformational changes of the proline conformation induced by the catalytical domain of Pin1 were followed by exchange spectroscopy. It was found that the different peptides do bind the Pin1 protein even better than the initially reported pThr231-Pro232 motif, and that at least the conformational exchange of the pThr212-Pro213 bond from *cis* to *trans* or *vice versa* can be catalyzed by Pin1. Because these results do not agree with the previously reported uniqueness of the pThr231-Pro231 site, we have looked into the possibility of other interaction sites on Tau, and confirmed that a minimal pThr-Pro dipeptide already can bind with a good affinity. These combined results show that the interaction of Pin1 and Tau is not limited to one unique site, and pose the intriguing question of a functional cooperativity between the WW and catalytic domain of Pin1 while it interacts with the hyperphosphorylated Tau.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Peptides were obtained by solid phase synthesis with introduction of selectively phosphorylated residues using appropriate building blocks of side chain-protected phosphothreonine or phosphoserine, as described previously (*33*). A dansyl fluorophore was added as a last step in the peptide synthesis of the pT212 peptide, by reaction of 5 equiv of dansyl chloride and 10 equiv of diisopropylethylamine in DMF. After TFA cleavage, peptides were purified by RP-HPLC on a C18 Nucleosil column (Macheey-Nagel, Duren, Germany) and eluted with an acetonitrile gradient. The homogeneity of the fractions was verified by MALDI-TOF mass spectrometry.

Expression and Purification of Recombinant Pin1 and the Catalytical Domain (Pin1$_{CAT}$). Pin1 with an N-terminal histidine tag fusion was produced in *Escherichia coli* strain BL21(DE3) (Novagen), carrying the recombinant PIN1-pET28 plasmid (generous gift from M. Yaffe, Harvard University, Cambridge, MA). The recombinant strain was grown at 37 °C in an LB medium containing kanamycin (20 mg/L) until the OD_{600} reached ~0.6. The cells were then harvested by centrifugation, and the pellet was resuspended in half the volume in M9 medium with $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Cambridge, MA) as the nitrogen source

[6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 4 g/L glucose, 1 g/L $^{15}NH_4Cl$, 0.12 g/L $MgSO_4$, 20 mg/L kanamycin, and MEM vitamin cocktail (Sigma)]. The culture was incubated at 37 °C for 1 h before induction with 0.5 mM IPTG followed by growth for 3 h at 31 °C. The cell pellet was resuspended in a lysis buffer [50 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0), 300 mM NaCl (buffer A), 10 mM imidazole, 1 mM DTT, 0.1% NP40, and a protease inhibitor cocktail (Roche)], and cell lysis was performed by sonication. The soluble extract was loaded on a nickel affinity column (Chelating Sepharose Fast Flow, Amersham Pharmacia Biotech). Unbound proteins were extensively washed with 20 mM imidazole in buffer A, and the protein of interest was eluted with 250 mM imidazole in buffer A.

The GST−$Pin1_{CAT}$ fusion protein was produced in the BL21(DE3) star strain using the pGEX-6P plasmid (Amersham Pharmacia Biotech). The production was performed in LB medium with 0.5 mM IPTG induction followed by incubation for 3 h at 31 °C before the cells were harvested. The pellet was resuspended in phosphate-buffered saline (PBS) [130 mM NaCl, 3 mM KCl, 10 mM $NaHPO_4$, and 2 mM KH_2PO_4 (pH 7.4)] containing 1% Triton X-100, 20 mM β-mercaptoethanol, and a protease inhibitor cocktail. GST fusion proteins were purified from the soluble extract by use of a GSTrap Chelating Sepharose column (Amersham) equilibrated in PBS (pH 7.2). Unbound proteins were extensively washed with PBS, and proteins were eluted after incubation with Precission Protease (Amersham) in 50 mM Tris (pH 8.0), 100 mM NaCl, and 4 mM EDTA at 4 °C for 20 h.

NMR Spectroscopy. NMR experiments were performed on a Bruker DMX spectrometer (Bruker, Karlsruhe, Germany) operating at 14.1 T and equipped with a cryogenic triple-resonance probe head. Spectral parameters were as specified in ref *33*. Standard TOCSY and NOESY experiments, with mixing times of 60 and 400 ms, respectively, were obtained with 1 or 2 mM peptide solutions in 50 mM deuterated Tris (pH 6.3) (Cambridge Isotope Laboratories) and 100 mM NaCl at 293 K. ^{15}N-labeled Pin1 HSQC spectra were recorded on a 200 μM protein sample in 50 mM deuterated Tris (pH 6.3), 100 mM NaCl, and a 5 mM DTT/EDTA mixture at the same temperature. For the titration experiments, a 200 μM solution of Pin1 was added to the appropriate amounts of the lyophilized peptide. The chemical shift perturbations of individual resonances calculated with the relationship $\Delta\delta$ (parts per million) = $[\Delta\delta^2(^1H) + 0.2\Delta\delta^2(^{15}N)]^{1/2}$ were used to derive the dissociation constants (*33*). For the observation of catalytic activity, 50 μM full-length Pin1 or its catalytic domain alone, each solubilized in D_2O buffer, was added to a 1.5 mM solution in D_2O of a triply phosphorylated peptide, and EXSY spectra with a mixing time of 400 ms were recorded.

Fluorescence Spectroscopy. FRET experiments were carried out on a PTI (Lawrenceville, NJ) fluorescence spectrometer by exciting tryptophan residues of Pin1 at 295 nm. A 20 μM solution of Pin1 in 50 mM Tris (pH 6.3) containing 100 mM NaCl and a 5 mM DTT/EDTA mixture was added to lyophilized aliquots of the peptide to obtain a full titration curve. When the dansylated peptides were used, the fluorescence quenching of the tryptophan indole groups by the dansyl moiety was assessed at 334 nm. The fluorescence variation was used to calculate the K_D as described previously (*33*).

Table 1: Peptide Sequences Synthesized during This Study[a]

peptide	sequence
T231	KKVAVVRT_{231}PPKSPSSAK
pT231	KKVAVVR***pT*****$_{231}$**PPKSPSSAK
pT231-pS235	KKVAVVR***pT*****$_{231}$**PPK***pS*****$_{235}$**PSSAK
T212	SRSRT_{212}PSLPTPPTR
pT212	SRSR***pT*****$_{212}$**PSLPTPPTR
pT212-pS214	SRSR***pT*****$_{212}$*****pS*****$_{214}$**LPTPPTR
pT212-pS214-pT217	SRSR***pT*****$_{212}$**P***pS*****$_{214}$**LP***pT*****$_{217}$**PPTR
pThr-Pro dipeptide	pTP

[a] Phosphorylated residues are in bold, and numbered according to their position in the 441-residue isoform. The peptides are represented in the text with the one-letter code, whereas the individual residues are represented with the three-letter code.

RESULTS

NMR Characterization of the Peptide Epitopes. Four different peptides were synthesized, corresponding to a nonphosphorylated sequence, and peptides containing the single pThr212 residue, pThr212/pSer214, and finally pThr212/pSer214/pThr217. As a control, we also synthesized sequences centered around Thr231, with phosphorylation of this latter site alone or of both Thr231 and Ser235 (Table 1).

Whereas assignment of such short peptides can now be routinely carried out according to the sequential assignment procedure based on combined TOCSY and NOESY spectra (*38*), the main problem for detecting and characterizing the minor *cis* forms proved to be less straightforward because of sensitivity reasons. We used 2 mM samples dissolved in 270 μL of aqueous buffer in H_2O or D_2O together with a cryogenic probe on a 600 MHz instrument to obtain good quality spectra of even the minor forms. The resulting spectra on all peptides show that phosphorylation has a large effect on the amide proton of the carrying Thr or Ser residue (*39*), making its ready detection from a one-dimensional spectrum feasible (Figure 1). The same phosphorylated residues followed by a Pro residue in the *cis* conformation show up as low-intensity peaks close to the H_N frequency of the major pThr/pSer signals followed by a Pro in the *trans* conformation.

When we first analyzed the peptides centered around the Thr231 residue, we confirmed the previously reported results for this epitope (*20*). The *cis* isomer of the pThr231-Pro232 peptide bond is populated at a level of <3% in water, and the conformation of the peptide as determined by NOE spectroscopy is essentially random. For the peptides centered around Thr212, confirmation of the Pro conformation came from the NOESY spectrum recorded in D_2O on both the non- and triphosphorylated peptides, where we observed specific NOE contacts between the Hα (and Hβ) protons of Thr212 and the Hα proton of *cis*-Pro213, and the same contacts between the corresponding protons of Thr217 and Pro218 (Figure 2). Other minor forms correspond to Pro216, which is preceded by a Leu, whereas no *cis* conformation could be detected for Pro219, despite the fact that its conformation could even be more constrained due to the preceding Pro residue.

Integration of the different NMR signals in the one-dimensional spectra or on the Hδ−Hδ NOE cross-peaks,

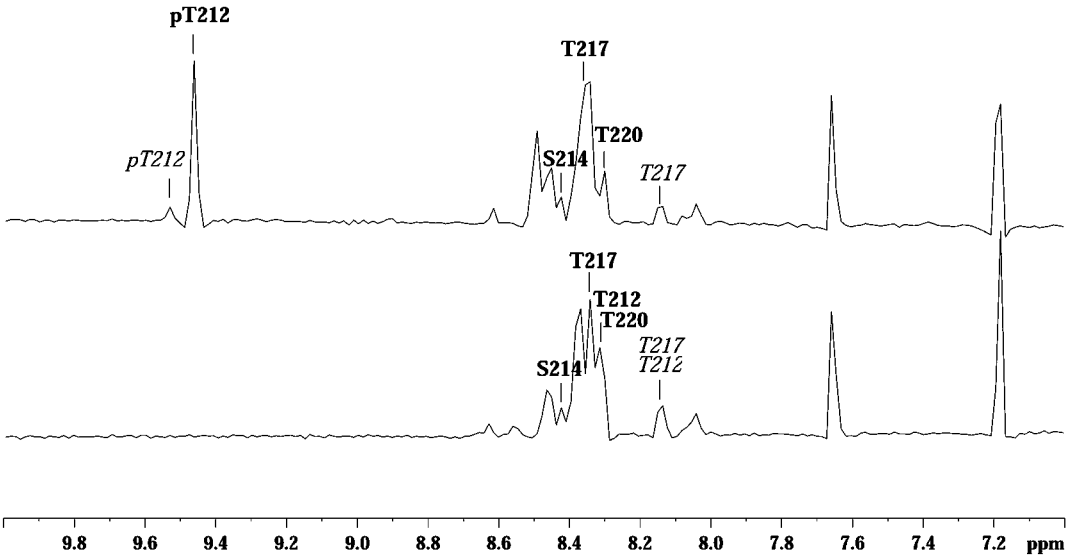

FIGURE 1: ^{1}H NMR spectra (zoom of the NH region) of the T212 peptide phosphorylated at the Thr212 position or unphosphorylated. Only Thr212, Ser214, and Thr217 are annotated. Those in bold precede a *trans*-Pro form, and those in italics precede a *cis*-Pro form.

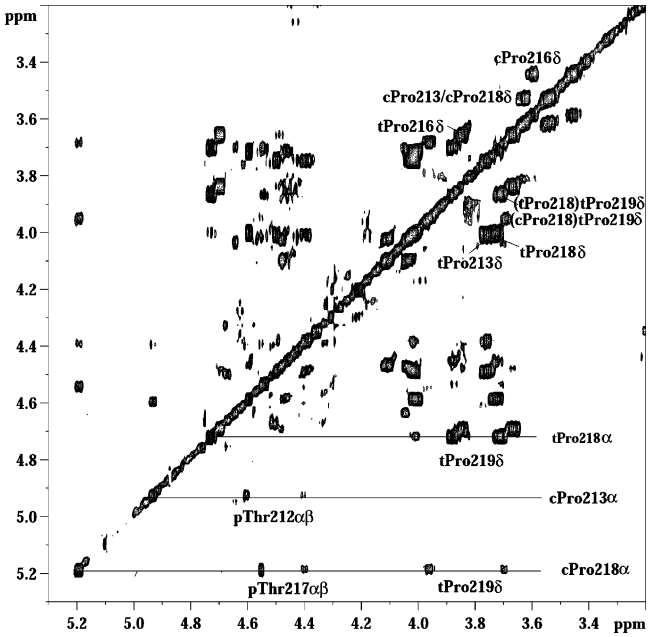

FIGURE 2: Assignment of the triphosphorylated T212 peptide on a 400 ms NOESY spectrum in D_2O. Hδ−Hα NOE contacts were assigned for *trans*-Pro isoforms and Hα−Hα for *cis*-Pro forms of Pro213 and Pro218. Hδ1−Hδ2 NOE contacts are annotated for all *trans*-Pro forms, for *cis*-Pro213, *cis*-Pro216, and *cis*-Pro218 forms, and for *trans*-Pro219 preceded by *cis*-Pro218.

Table 2: Relative Populations of the *cis*/*trans* Ratio for the Different Pro Residues in the Non- or Triphosphorylated T212 Peptide

	cis/*trans* ratio (%)			
peptide	P213	P216	P218	P219
T212	6	8	12	<1
pT212-pS214-pT217	7	8	13	<1

where a reasonable dispersion exists, allowed the estimation of the relative populations of the *trans* and *cis* conformers (Table 2). Importantly, it was found that even a triple phosphorylation did not significantly change the relative populations.

Interaction Mapping of the Peptides with Pin1. Rather than using the isolated WW domain as we reported in a previous NMR study (*33*), we decided to work directly with the full-length ^{15}N-labeled Pin1 protein. Near-complete assignments for this protein were previously reported (*40*), enabling a direct identification of the WW resonances in the full-length protein. When we titrated the phosphopeptide centered around the Thr231 epitope against a constant concentration of full-length Pin1, we found a nearly identical value for the dissociation constant. A similar titration experiment with a nonphosphorylated peptide did not yield detectable binding. When we repeated the experiment with the pT212 peptide, carrying a single phospho group on the Thr212 side chain, we detected a 3-fold stronger interaction, confirming that pThr231 is neither a unique nor even the best binding epitope. Resonances on the Pin1 protein that shifted upon addition of the pT212 peptide were mainly assigned to the WW domain, with the strongest shifts for the same residues (Ser16−Gly20 and Gln33−Glu35, with a very pronounced variation for the Trp34 HNε1 side chain moiety) that were identified in the titration with the pT231 peptide (*33*). Small variations were equally detected for some resonances on the catalytic domain. A first category of those was assigned to residues at the interface of the WW and catalytical domain, including, for example, the Phe139 and Leu141 residues. On the basis of the chemical shift variation of their resonances as a function of the increasing peptide concentration, we derived a K_D constant similar to the one describing the interaction with the WW domain (Table 4). A second group of resonances that shift slightly upon addition of peptides are those of the catalytic site, including Met130, Leu122, Ser114, and Ser115. Because even at the 20-fold peptide excess used in our titration study, saturation of the chemical shift variations was not attained, we could not derive a reliable K_D value for the catalytic domain. This very weak interaction is in agreement with our findings for the interaction between phosphopeptides and the Pin1 protein of *Arabidopsis thaliana*, which consists only of a catalytic domain (*41*).

Table 3: Chemical Shift Values of the Triply Phosphorylated pT212-pS214-pT217 Peptide[a]

				Hβ		Hγ		Hδ	
		H_N	Hα	Hβ1	Hβ2	Hγ1	Hγ2	Hδ1	Hδ2
208	S	8.38	4.43	3.87					
209	R	8.5	4.44	1.91	1.79	1.67		3.21	
210	S	8.49	4.45	3.89					
211	R	8.48	4.37	1.91	1.79	1.67		3.23	
212 (t)	pT	9.45	4.50	4.40		1.38			
212 (c)	pT	9.52	4.61	4.41		1.27			
213 (t)	P	−	4.43	2.34	2.09	2.04	1.93	4.01	3.78
213 (c)	P	−	4.94	2.41	2.12	1.95	1.84	3.61	3.48
214	pS	9.10	4.44	4.11	3.98				
215 (t)	L	8.40	4.69	1.71	1.63	1.59		0.97	0.94
215 (c)	L	7.80	4.51	1.60		1.43		0.91	
216 (t)	P	−	4.49	2.31	2.03	1.89		3.87	3.68
216 (c)	P	−	4.60	2.41	2.13	1.95	1.85	3.62	3.48
217 (t)	pT	9.03	4.60	4.47		1.37			
217 (c)	pT	9.20	4.56	4.40		1.30			
218 (t)	P	−	4.72	2.4	2.09	2.05	1.9	4.01	3.75
218 (c)	P	−	5.20	2.52	2.17	2.00	1.86	3.62	3.54
219	P	−	4.55	2.36	2.09	2.05	1.93	3.89	3.71
220	T	8.29	4.32	4.22		1.22			

[a] Minor form assignments of residues in the peptide with a Pro in the *cis* conformation are given.

Table 4: Dissociation Constant Values Obtained by NMR for Various Phosphorylated Peptide Substrates against the Full-Length Pin1 Protein[a]

peptide	K_D^{PIN1} (mM)	peptide	K_D^{PIN1} (mM)
pT231	0.38 ± 0.1	pT212-pS214	0.16 ± 0.02
pT231-pS235	0.18 ± 0.05	pT212-pS214-pT217	0.10 ± 0.01
pT212	0.10 ± 0.03	pThr-Pro dipeptide	0.15 ± 0.03

[a] NMR dissociation constant values are based on chemical shift perturbations of several residues ($N > 5$). The data for the dipeptide were obtained with the isolated WW domain.

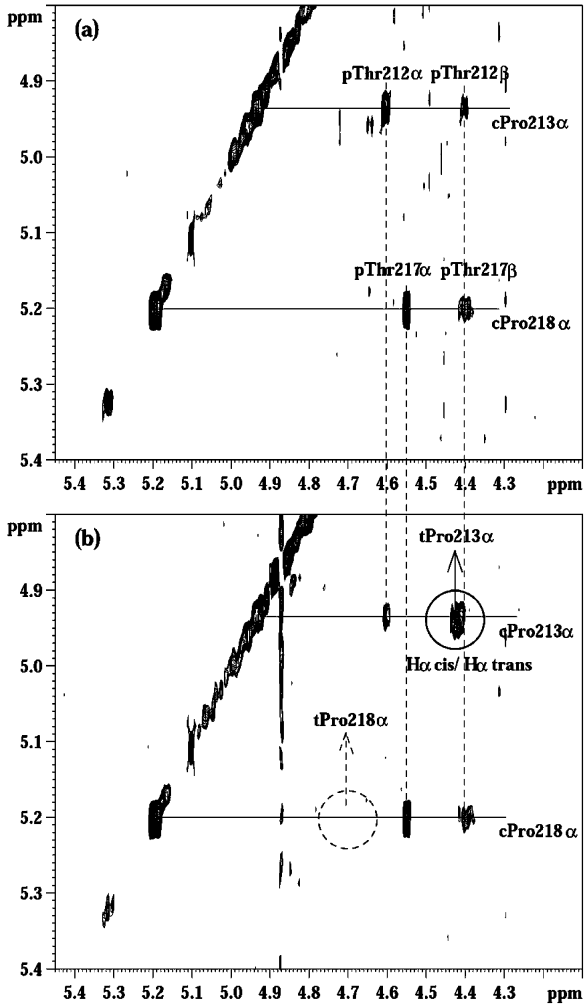

FIGURE 3: (a) Region around Hα *cis*-Pro213 and Pro218 in the 400 ms EXSY spectrum of the triphosphorylated T212 peptide. Correlations involving pThr212 and pThr217 Hα and Hβ minority forms are annotated. (b) Same region in the 400 ms EXSY spectrum of the triphosphorylated T212 peptide containing 50 μM Pin1 showing an additional signal that correlated Hα *cis* and Hα *trans* of Pro213 (circled). The corresponding signal for Pro218 is not observed as indicated by the dashed circle.

To investigate the influence of multiple phosphorylations on the interaction, we performed similar titration experiments with three different peptides, including doubly phosphorylated Alzheimer-type epitopes pThr231/pSer235 and pThr212/pSer214. Amazingly, the effect on the K_D for Pin1 of a second phosphorylation is not the same for both peptides; the addition of a phosphate on Ser235 increases the affinity by a factor of 2, whereas phosphorylation of Ser214 decreases the affinity for Pin1. Addition of a third phosphorylation site to the latter peptide (Thr217) was necessary to restore the initial dissociation constant of 100 μM.

The dissociation constant values for the pT212 peptide−Pin1 interaction were confirmed in an independent experiment based on the fluorescence variation of Pin1 upon titration in a dansylated pT212 peptide. Because of energy transfer from the tryptophan emitting at 334 nm to the dansyl group on the N-terminus of the pT212 peptide, the fluorescence of the tryptophans at 334 nm is efficiently quenched (Figure 4a). The resulting decrease in fluorescence intensity can more readily be quantified as the increase in the intrinsic tryptophan fluorescence that we had previously used with a nondansylated peptide as a substrate (*42*). The K_D value of 110 μM confirms the data from the NMR titration experiment (Figure 4b), and demonstrates the absence of interference by the dansyl group.

Catalytic Activity of Pin1. The absence of a detectable amount of the *cis* form in the pThr231 peptide makes it a less suitable substrate for the detection of a potential catalytic action of Pin1. Because the situation with the epitope centered around pThr212 is different, and because the use of the cryogenic probe had allowed the full assignment of all minor *cis* forms (Table 3), we used EXSY spectroscopy to probe the catalytic activity of Pin1. The experimental setup was similar to the one we used to demonstrate the catalytic activity of the *A. thaliana* Pin1 protein (*35*), with 400 ms EXSY spectra of the triply phosphorylated peptide in D_2O with or without Pin1 added. Without Pin1, the isomerization proved to be too slow to give detectable cross-peaks connecting the signal of the same proton on a peptide in two conformations. When we added a catalytic amount of the protein, however, several additional cross-peaks appeared. The contact between the Hα protons of Pro213 in the *cis* and *trans* conformations was easily identified (Figure 3), despite the presence of a weak contact between the Hα proton of Pro213 in the *cis* conformation and the Hβ proton of pThr212. The structural transition of the Thr212-Pro213 amide bond was further confirmed by a contact between the Hδ protons of Pro213 in the *cis* and *trans* conformations.

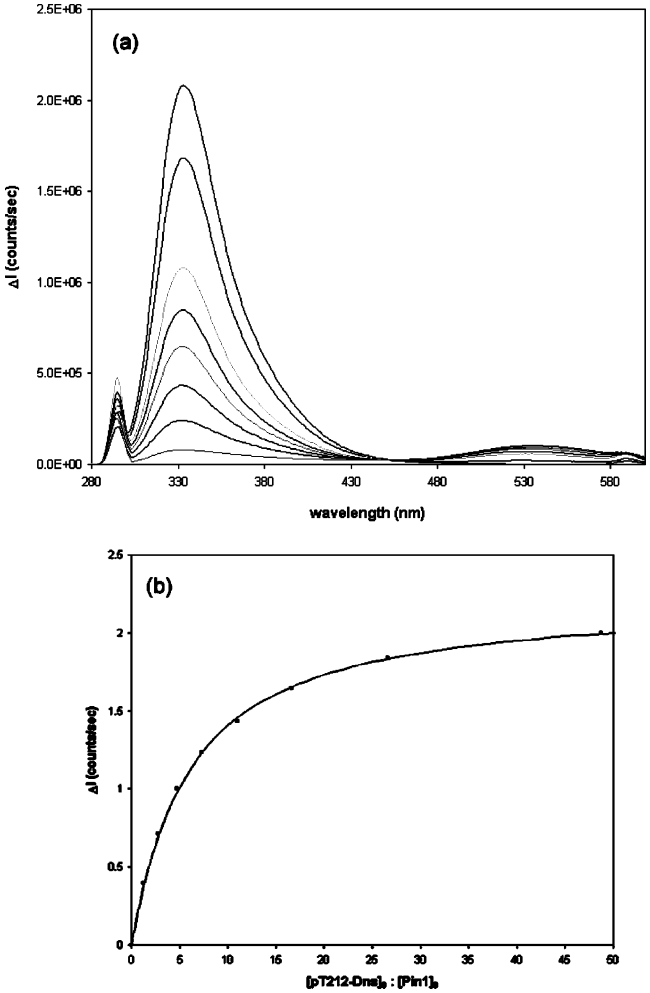

FIGURE 4: (a) Fluorescence titration of Pin1 by the pT212 Tau peptide coupled to a dansyl group at the N-terminus (pT212-Dns) involving FRET. Increasing amounts of the peptide were added to a 20 μM solution of Pin1, resulting in a quenching of the fluorescence signal at 334 nm and a slight increase in the dansyl signal at 540 nm. (b) Graphical representation of the K_D calculation for the pT212 peptide based on the intensity variations of the fluorescence at 334 nm plotted against the molar ratio of the pT212 dansylated peptide to Pin1 concentration.

The isolated character of the Hα proton of Pro218 in the *cis* conformation should make the observation of an exchange-mediated contact straightforward, but even with a long mixing time of 400 ms, we could not observe such a contact. This indicates that Pin1 selectively catalyzes the conformational change of the peptide bond between pThr212 and Pro213, but not the one between pThr217 and Pro218. When we performed the same experiment with the isolated catalytic domain of Pin1, very similar results were obtained.

DISCUSSION

The phosphorylation of Tau has been the subject of intense studies. A first line of research was aimed at the mapping of the *in vivo* phosphorylated sites of fetal or adult soluble Tau (*3*) as well as the Tau found in the insoluble PHF aggregates (*7*, *8*). Both immunochemical and protein chemistry techniques led to the conclusion that adult Tau carries significantly fewer phosphates than fetal Tau, and that phosphorylation on PHF Tau is even more pronounced than in fetal Tau. The dual character of the hyperphosphorylation stems from the fact that a larger number of phosphorylation sites has been identified in PHF Tau than in fetal Tau, and second, the occupation level of the individual sites is higher in PHF Tau than in fetal Tau. An example of this latter factor is the Thr231 site, which is only phosphorylated to a level of 30% in fetal Tau, whereas no unphosphorylated Thr231 is found in PHF Tau (*8*).

Whereas phosphorylation of the Ser262 residue or Ser214 is known to promote detachment of Tau from the microtubules, and might hence lead to microtubule destabilization (*13*), a clear relationship between phosphorylation and aggregation has not been established. Recent results, for example, have demonstrated that phosphorylation of the same Ser262 site actually prevents aggregation (*17*), in apparent contradiction with the initial hypothesis that hyperphosphorylation is a causal factor for aggregation. The sole factor of phosphorylation might thus not be sufficient to explain the physicochemical changes that govern microtubule interaction or tangle formation. Additional evidence for this comes from the recent finding that the perturbed interaction between phosphorylated Tau and tubulin might be restored by the parvulin Pin1 (*23*). This latter enzyme belongs to the class of prolyl *cis*/*trans*-isomerases that catalyze the conformation of the prolyl bond. However, whereas the cyclophilins or FKBP binding proteins, the two main other classes of prolyl *cis*/*trans*-isomerases, are at least in yeast not essential for correct cell functioning (*43*), Pin1 or its yeast analogue Ess1 is crucial for correct progress through the cell cycle. Many of the recognition motifs of Pin1 coincide moreover with the epitopes of the monoclonal MPM2 antibody. This latter recognizes mitosis specific phosphoproteins, confirming further the role of Pin1 in the cell cycle.

Pin1 is characterized by a specificity at the substrate level, as it catalyzes the conformational change only when the proline is preceded by a phosphorylated Thr or Ser residue. The same motif is recognized by the second structural unit, the 40-amino acid WW domain, that seemingly promotes protein−protein interactions and is essential for the correct functioning of the protein (*44*). The interaction of Pin1 with a large number of phosphoproteins involved in the cell cycle has led to the hypothesis of a second conformational mechanism of posttranslational control after the covalent modification of phosphorylation. A proline *cis*/*trans* isomerization could be the structural switch, but direct evidence of such a mechanism with true protein targets has only very recently begun to emerge (*45*). Finally, it should be noted that the described interaction sites on several key mitotic proteins are in the unstructured regulatory parts of the protein. Examples are the Thr48 and Thr67 sites on the regulatory part of CDC25C (*46*, *47*), or the Ser63 and Ser73 epitopes on c-JUN (*48*).

Because phosphorylated Tau extracted from the brains of people with AD is equally recognized by the mitotic MPM2 antibodies (*49*), Lu et al. (*23*) set out to study its interaction with Pin1. They discovered several intriguing aspects. First, Pin1 would be able to restore the microtubule binding capacity of Cdc2/cyclin B-phosphorylated Tau, and second, the interaction with phospho-Thr231 would be necessary and sufficient for this interaction. However, the observed specificity is not easy to explain at the structural level, as several studies have proven that the main interaction motif of the WW domain is the pThr-Pro dipeptide, with a minor contribution of the residue following the Pro (*32*, *33*).

Moreover, although Thr231 is indeed phosphorylated to a higher level in PHF Tau, it can at least partially be phosphorylated in fetal Tau, raising the question of the conformational specificity of the Pin1−Tau interaction. For these reasons, we decided to further investigate the uniqueness of the interaction.

Of those sites that are unique to PHF Tau, two (Thr212 and Ser422) are proline-directed sites. We decided to first investigate the former site, because it is in the vicinity of the Thr231 residue, with the same distance from the latter as the two epitopes described on CDC25C (*34*). Moreover, because this Thr212 residue has at least two other phosphorylation sites in its immediate vicinity, we synthesized the peptides in different combinations of phosphorylation. NMR spectroscopy was used to investigate first the conformational aspects of those peptides, with specific attention being paid to the *cis*/*trans* ratios of the different prolines that are involved. Indeed, the absence of interaction between one of those epitopes and Pin1 could be due to the latter containing a Pro residue completely constrained in the *cis* conformation, where interaction with the WW domain is no longer possible (*32*, *33*). A previous study with peptides centered around Thr231 had shown that the phospho-Thr−Pro bond in this peptide is predominantly in the *trans* conformation, with only 3% in the *cis* conformation. Addition of TFE, a solvent that is supposed to mimic the more hydrophobic environment of the inner protein, even further reduced this fraction (*20*). Therefore, it is not clear how a local structural change in such a small amount of the *cis* conformation containing chains could form the structural switch that explains the functional interaction between Pin1 and Tau. Of course, our results are obtained on a small peptide substrate and hence do not necessarily reflect the situation in the full-length protein. Still, recent NMR results of our group tend to confirm that the random coil chemical shift can be used efficiently to assign at least partially the full-length protein (*51*), confirming its unstructured nature and validating the peptide approach.

In our T212 peptide, the pool of peptide bonds in the *cis* conformation is somewhat larger, and this occurs not only at the level of the Thr212-Pro213 bond. Indeed, using a 2 mM sample and a measuring time of 12 h on a 600 MHz spectrometer equipped with a cryoprobe, we were able to distinguish the conformational heterogeneity at the level of the Pro213, Pro216, and Pro218 peptide bonds, and assign the different peptides in solution (Figure 2). On the basis of the integration of the one-dimensional proton spectrum and of the proline $\delta1-\delta2$ cross-peaks in the NOESY spectrum, we obtained the relative concentrations for the *trans* and *cis* peptide bonds preceding Pro213, Pro216, and Pro218 (Table 1). Only for Pro219 could we not detect a *cis* conformation, which limits its level to an estimated 1%. However, when comparing the relative populations in the differently phosphorylated peptides, we conclude that there is no clear modification of the *cis*/*trans* distribution with an increasing level of phosphorylation. As for the secondary structure of the peptides, the only noteworthy NOE contact that we observed was a medium-intensity contact between the amide protons of Ser214 and Leu215. This might indicate a kink in the peptide structure, but it remains to be seen whether this contact is an artifact of the short size of the peptide or whether it is truly present in the full-length protein.

The interaction of the different peptides with Pin1 was first investigated in a series of titration experiments against full-length Pin1. The ^{15}N-labeled protein was used simultaneously to derive the dissociation constant from a fit of the chemical shift variations as a function of increased peptide concentration and to map those residues that directly interact with the peptide. A first titration experiment with the pT231 peptide resulted in a similar K_D value as previously determined against the isolated WW domain (*33*), confirming the primordial role of the WW domain as a protein−protein interaction module. When we performed a second titration series with a peptide phosphorylated at both Thr231 and Ser235, mimicking thereby the recognition site of the Alzheimer specific TG3 antibody, the affinity increased roughly 2-fold. We are at present using calorimetry to study whether this increased affinity results in a loss of entropy in the unbound state, or from a true increase in the enthalpy of binding.

The main result of our study came when we used the pT212 peptide in the same titration study. Indeed, the affinity of this peptide proved to be even better than that of the peptide centered on pThr231. Because this result casts doubt on the proposed uniqueness of this interaction site, we repeated the same measurement by fluorescence spectroscopy with a peptide modified with a dansyl group at its N-terminus. Energy transfer between Tryp34 of the WW domain and the dansyl acceptor proved to be very efficient, and the resulting fluorescence decrease proved to be easier to interpret than the intrinsic fluorescence changes that we had previously used to confirm independently the NMR K_D value for the pT231−WW interaction (*42*). Despite the addition of a dansyl, the resulting K_D value confirmed our previous results derived by NMR.

Contrary to the case of the pT231 peptide, a second phosphorylation on the Ser214 site decreases the affinity by a factor of 2. We can rationalize this observation with the crystal structure of Pin1 in complex with a peptide derived from the C-terminal domain of polymerase II, where the hydroxyl Ser residue downstream of the phospho-Thr-Pro recognition motif also makes a hydrogen bond with the WW Trp34 side chain (*32*). In the pT212 peptide, we also find a Ser214 residue, and from the fact that the affinity of pT212 is higher than that of the pT231 peptide, where the Pro233 side chain does not offer the possibility of a hydrogen bond, we can safely assume that Ser214 indeed contributes to the increased affinity. Phosphorylation, however, would introduce a bulky phosphate group, which might be harder to fit than the simple hydroxyl group. Only when we create a second canonical phospho-Thr217-Pro218 interaction motif in the triply phosphorylated peptide did we find our initial dissociation constant of 100 μM, indicating that both dipeptides are recognized with similar affinities. This latter result motivated us to measure the affinity of the isolated phospho-Thr-Pro dipeptide, devoid of any sequence context. The dissociation constant measured is on the same order of magnitude as that for the pT212 peptide, confirming that the main motif of interaction is indeed the dipeptide, and that the other amino acids only weakly affect this binding.

Because the interaction mapping studies were performed with the full-length Pin1 protein, we could also observe potential interaction sites with the catalytic domain. A first category of residues whose amide correlation shifted slightly

upon addition of the peptide includes residues at the interface between the WW and catalytic domain. Because the K_D derived from those chemical shift variations is identical to the one obtained for the WW domain, we conclude that it concerns the same binding event, and that binding of the peptide to the WW domain slightly interferes with the catalytic domain. This is in agreement with two recent NMR studies, where chemical shift mapping combined with interdomain NOEs and residual dipolar couplings led to the conclusion of possible cross-talk between the two domains, although the fine details seem to be substrate-dependent (*50*, *51*). An independent interaction site, be it with a very weak affinity, was identified in the long loop that was found to contain the Ala-Pro dipeptide in the first crystal structure (*25*). However, as was the case for Pin1 from *A. thaliana*, only a lower bound of 10 mM could be determined for the affinity constant.

On the basis of the fact that both *cis* and *trans* conformations can be observed for Pro213 and Pro218 in this triphosphorylated peptide, we have investigated the Pin1 catalytic activity by adding 50 μM enzyme to a 1.5 mM solution of the peptide in D_2O. Unexpectedly, the only Pin1-dependent prolyl *cis*/*trans* isomerization detected through an *cis*-Hα−*trans*-Hα exchange cross-peak was for the Pro213 residue. A similar correlation peak was not observed for the Pro218 residue, although this motif is characterized by twice as much *cis* conformer as the peptide bond preceding Pro213. The presence of a Pro residue before and after the pThr217-Pro218 moiety possibly imposes a structural constraint such that the *cis*/*trans* interconversion can no longer be catalyzed by the Pin1 enzymatic domain.

Phosphorylation of the different Thr/Ser-Pro sites of the Tau protein is controlled by various well-known kinase−phosphatase complexes. Phosphorylation can be implemented among others *in vitro* with purified activated kinases such as the Cdc2−cyclin B complex used in the initial study identifying the Pin1−Tau interaction (*23*). However, this latter kinase was previously shown to incorporate only 0.1 phosphate unit per Tau molecule, even with an incubation time of 24 h (*29*). This latter factor might explain why the interaction with other sites such as the Thr212-Pro213 site described here initially went undetected, as the Cdc2−cyclin B complex probably only slightly catalyzes, if at all, the phosphorylation of this site (*41*). This is of course in sharp contrast to the peptide studies, where chemical synthesis allows for a 100% incorporation of a phosphate at any given position. As for the important question of whether T212 can interact with Pin1 in the full-length protein, preliminary pull-down results with recombinant Tau phosphorylated by a COS cell extract in the presence of the PP2A phosphatase blocking okadaic acid show that the T231A mutant and the T212A mutant still interact with Pin1 (A.-V. Sambo, M. Hamdame, and L. Buée, unpublished results). The double mutation (T212A/T231A) leads to a further decrease in the level of the retained protein, but still not a complete absence of interaction, suggesting the presence of even other interaction sites.

In conclusion, we have shown through careful peptide mapping that the phospho-Thr231-Pro232 dipeptide is not the only interaction motif between Tau and the cell cycle specific prolyl *cis*/*trans*-isomerase Pin1, and that the phospho-Thr212-Pro213 motif has an even higher affinity for the Pin1 WW domain. The enzymatic activity of Pin1 on this latter site was confirmed through exchange NMR spectroscopy. Many questions about the molecular details of the Pin1−Tau interaction remain, however, and one of the most important is the possible cooperativity between both Thr212 and Thr231 sites, which could be a model for the action of Pin1 on other substrates such as Cdc25C. Finally, all our work is related to the interaction between Pin1 and Tau with the latter in its phosphorylated but free state. In the microtubule-bound state, however, we cannot exclude the possibility that one of several phosphorylated Thr/Ser-Pro motifs of Tau is forced into a *cis* conformation, and that those motifs are the true substrates for the prolyl *cis*/*trans*-isomerase activity of Pin1.

Future efforts will be directed to larger substrates encompassing both sites, in both their free and microtubule-bound state, and should be able to describe in further detail the intriguing functional role of the WW and prolyl isomerase domains of Pin1.

REFERENCES

1. Delacourte, A., and Buée, L. (1997) *Int. Rev. Cytol. 171*, 167−224.
2. Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D., and Crowther, R. A. (1989) *Neuron 3*, 519−526.
3. Watanabe, A., Hasegawa, M., Suzuki, M., Takio, K., Morishima-Kawashima, M., Titani, K., Arai, T., Kosik, K. S., and Ihara, K. Y. (1993) *J. Biol. Chem. 268*, 25712−25717.
4. Biernat, J., Mandelkow, E.-M., Schröter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Mercken, M., Vandermeeren, A., Goedert, M., and Mandelkow, E. (1992) *EMBO J. 11*, 1593−1597.
5. Otvos, L., Feiner, L., Lang, E., Szendrei, G. I., Goedert, M., and Lee, V. M.-Y. (1994) *J. Neurosci. Res. 39*, 669−673.
6. Goedert, M., Jakes, R., Crowther, R. A., Cohen, P., Vanmechelen, E., Vandermeeren, M., and Cras, P. (1994) *Biochem. J. 301*, 871−877.
7. Hasegawa, M., Morishima-Kawashima, M., Takio, K., Suzuki, M., Titani, K., and Ihara, Y. (1992) *J. Biol. Chem. 267*, 17047−17054.
8. Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Watanabe, A., Titani, K., and Ihara, Y. (1995) *Neurobiol. Aging 16*, 365−371.
9. Brandt, R., Lee, G., Teplow, D. B., Shalloway, D., and Abdel-Ghany, M. (1994) *J. Biol. Chem. 269*, 11776−11782.
10. Wang, J. Z., Wu, Q., Smith, A., Grundke-Iqbal, I., and Iqbal, K. **(**1998) *FEBS Lett. 436*, 28−34.
11. Godemann, R., Biernat, J., Mandelkow, E., and Mandelkow, E.-M. (1999) *FEBS Lett. 454*, 157−164.
12. 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.
13. Gustke, N., Steiner, B., Mandelkow, E. M., Biernat, J., Meyer, H. E., Goedert, M., and Mandelkow, E. (1992) *FEBS Lett. 307*, 199−205.
14. Vincent, I., Rosado, M., and Davies, P. (1996) *J. Cell Biol. 132*, 413−425.
15. Vincent, I., Zheng, J. H., Dickson, D. W., Kress, Y., and Davies, P. (1998) *Neurobiol. Aging 19*, 287−296.
16. 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*, 1−9.
17. Schneider, A., Biernat, J., von Bergen, M., Mandelkow, E., and Mandelkow, E. M. (1999) *Biochemistry 38*, 3549−3558.
18. Hoffmann, R., Lee, V. M., Leight, S., Varga, I., and Otvos, L., Jr. (1997) *Biochemistry 36*, 8114−8124.
19. Jicha, G. A., Lane, E., Vincent, I., Otvos, L., Jr., Hoffmann, R., and Davies, P. (1997) *J. Neurochem. 69*, 2087−2095.
20. Daly, N. L., Hoffmann, R., Otvos, L., Jr., and Craik, D. J. (2000) *Biochemistry 39*, 9039−9046.
21. Lu, K., Hanes, S., and Hunter, T. (1996) *Nature 380*, 544−547.

22. Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J. U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., and Lu, K. P. (1997) *Science 278*, 1957−1960.
23. Lu, P.-J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) *Nature 399*, 784−788.
24. Stukenberg, P. T., and Kirschner, M. W. (2001) *Mol. Cell 7*, 1071−1083.
25. Ranganathan, R., Lu, K., Hunter, T., and Noel, J. (1997) *Cell 89*, 875−886.
26. Sudol, M., Bork, P., Einbond, A., Kastury, K., Druck, T., Negrini, M., Huebner, K., and Lehman, D. (1995) *J. Biol. Chem. 270*, 14733−14741.
27. Fischer, G., and Aumuller, T. (2003) *Rev. Physiol., Biochem. Pharmacol. 148*, 105−150.
28. Ishiguro, K., Shiratsuchi, A., Sato, S., Omori, A., Arioka, M., Kobayashi, S., Uchida, T., and Imahori, K. (1993) *FEBS Lett. 325*, 167−172.
29. Drewes, G., Lichtenberg-Kraag, B., Doring, F., Mandelkow, E. M., Biernat, J., Goris, J., Doree, M., and Mandelkow, E. (1992) *EMBO J. 11*, 2131−2138.
30. Paudel, H. K., and Li, W. (1999) *J. Biol. Chem. 274*, 8029−8038.
31. Vulliet, R., Halloran, S. M., Braun, R. K., Smith, A. J., and Lee, G. (1992) *J. Biol. Chem. 267*, 22570−22574.
32. Verdecia, M. A., Bowman, M. E., Lu, K. P., Hunter, T., and Noël, J. P. (2000) *Nat. Struct. Biol. 7*, 639−643.
33. Wintjens, R., Wieruszeski, J.-M., Rousselot-Pailley, P., Drobecq, H., Lippens, G., and Landrieu, I. (2001) *J. Biol. Chem. 276*, 25150−25156.
34. Crenshaw, D., Yang, J., Means, A., and Kornbluth, S. (1998) *EMBO J. 17*, 1315−1327.
35. Landrieu, I., De Veylder, L., Fruchart, J. S., Odaert, B., Casteels, P., Portetelle, D., Van Montagu, M., Inze, D., and Lippens, G. (2000) *J. Biol. Chem. 275*, 10577−10581.
36. Lefebvre, T., Ferreira, S., Dupont-Wallois, L., Bussiere, T., Dupire, M. J., Delacourte, A., Michalski, J.-C., and Caillet-Boudin, M. L. (2003) *Biochim. Biophys. Acta 1619*, 167−176.
37. Mailliot, C., Bussiere, T., Caillet-Boudin, M. L., Delacourte, A., and Buée, L. (1998) *Neurosci. Lett. 255*, 13−16.
38. Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley-Interscience, New York.
39. Bienkiewicz, E. A. (1999) *J. Biomol. NMR 15*, 203−206.
40. Jacobs, D. M., Saxena, K., Grimme, S., Vogtherr, M., Pescatore, B., Langer, T., Elshorst, B., and Fiebig, K. M. (2002) *J. Biomol. NMR 23*, 163−164.
41. Landrieu, I., Wieruszeski, J.-M., Wintjens, R., Inze, D., and Lippens, G. (2002) *J. Mol. Biol. 320*, 321−332.
42. Hamdane, M., Smet, C., Sambo, A.-V., Leroy, A., Wieruszeski, J. M., Delobel, P., Maurage, C. A., Ghestem, A., Wintjens, R., Bégard, S., Sergeant, N., Delacourte, A., Horvath, D., Landrieu, I., Lippens, G., and Buée, L. (2002) *J. Mol. Neurosci. 19*, 275−287.
43. Dolinski, K., Muir, S., Cardenas, M., and Heitman, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 13093−13098.
44. Lu, P. J., Zhou, X. Z., Shen, M., and Lu, K. P. (1999) *Science 283*, 1325−1328.
45. Andreotti, A. (2003) *Biochemistry 42*, 9515−9524.
46. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. **(**1998) *Genes Dev. 12*, 706−720.
47. 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.
48. Kondratick, C. M., and Vandre, D. D. (1996) *J. Neurochem. 67*, 2405−2416.
49. Bayer, E., Goettsch, S., Mueller, J. W., Griewel, B., Guiberman, E., Mayr, L. M., and Bayer, P. (2003) *J. Biol. Chem. 278*, 26183−26193.
50. Jacobs, D. M., Saxena, K., Vogtherr, M., Bernado, P., Pons, M., and Fiebig, K. (2003) *J. Biol. Chem. 20*, 26174−26182.
51. Lippens, G., Wieruszeski, J.-M., Leroy, A., Smet, C., Sillen, A., Buée, L., and Landrieu, I. (2004) *ChemBioChem 4*, 100−105.

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