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**Neurofibrillary Tangles as a Target
for Developing New Therapeutics
for Alzheimer's Disease**

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Pin1

A Therapeutic Target in Alzheimer Neurodegeneration

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Abstract

In Alzheimer's disease, the peptidyl prolyl cis/trans isomerase Pin1 binds to phospho-Thr231 on Tau proteins and, hence, is found within degenerating neurons, where it is associated to the large amounts of abnormally phosphorylated Tau proteins. Conversely, Pin1 may restore the tubulin polymerization function of these hyperphosphorylated Tau. In the present work, we investigated, both at the cellular and molecular levels, the role of Pin1 in Alzheimer's disease through the study of its interactions with phosphorylated Tau proteins. We also showed that in neuronal cells, Pin1 upregulates the expression of cyclin D1. This, in turn, could facilitate the transition from quiescence to the G1 phase (re-entry in cell cycle) in a neuron and, subsequently, neuronal dedifferentiation and apoptosis. The involvement of Pin1 in the G0/G1 transition in neurons points to its function as a good target for the development of new therapeutic strategies in neurodegenerative disorders.

Index Entries: Tau proteins; NMR spectroscopy; cell cycle; phosphorylation; rotamase.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that leads to dementia and affects approx 10% of the population older than 65 yr of age. Memory loss is the first sign of cognitive impairment, followed by aphasia, agnosia, apraxia, and behavioral disturbances. The two main types of brain lesion observed in AD are amyloid deposits and neurofibrillary tangles (NFTs). Amyloid deposits result from the extracellular accumulation of a peptide referred to as A β into amyloid deposits. A β

derives from a precursor, the β -amyloid precursor protein (APP). In cases of familial AD, mutations have been found on the APP gene, suggesting that it plays a central role in the etiopathogenesis. NFTs correspond to the aggregation of abnormal fibrils into paired helical filaments (PHFs), within certain vulnerable neuronal populations. The major antigenic components of PHFs are abnormally phosphorylated Tau proteins. In the adult human brain, Tau proteins are found essentially in neurons. They play important roles in the polymerization and stability of microtubules. At least 30 phosphorylation

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sites have been described in Tau proteins, most of which occur on Ser/Pro and Thr/Pro motives. The effect as a result of the phosphorylation of each of these sites is not known, but it is well established that phosphorylation decreases the efficiency of Tau proteins to promote microtubule polymerization (for review, *see* Buée et al., 2000). Aggregation of microtubule-associated Tau proteins into filaments is a common feature encountered in AD and other neurodegenerative disorders referred to as tauopathies. Abnormal phosphorylation is the major modification of these proteins aggregated into intracellular filamentous inclusions (Buée et al., 2000). For instance, phosphorylation-dependent antibodies such as TG3 that recognize conformation-dependent epitopes can visualize phosphorylated Tau aggregated into filaments. The TG3 epitope (phosphorylated Thr231) is expressed in mitotic cells but not in quiescent cells (Vincent et al., 1996), suggesting that mitotic phospho-epitopes may lead to conformational changes (Jicha et al., 1997; Delobel et al., 2002). Re-entry of the neuron in cell cycle is one of the mechanisms that activates anarchic transduction pathways leading to the abnormal phosphorylation of Tau proteins and their aggregation in tauopathies (Delobel et al., 2002; Nagy, 2000).

Pin1 is a recently characterized human enzyme belonging to the class of peptidyl-prolyl cis/trans isomerase (PPIase) and, more specifically, to the subclass of parvulins (Lu et al., 1996). It catalyzes the cis/trans isomerization of the proline-preceding peptide bond, which is an intrinsically slow process. Pin1 preferentially recognizes substrates with a phosphorylated serine or threonine (pSer/pThr) N-terminal at the proline residue (Yaffe et al., 1997; Ranganathan et al., 1997) and, via an N-terminal WW domain, interacts with various phosphoproteins through the same pSer/pThr-Pro motif (Lu et al., 1999a). This binding activity is required for Pin1 to interact with its substrate *in vitro* and to perform its essential function *in vivo* (Shen et al., 1998; Lu et al., 1999a).

In a first crystal structure of Pin1 (Ranganathan et al., 1997), structural evidence for the substrate specificity came from the presence of a sulfate molecule next to an Ala-Pro dipeptide in the crystal. The sulfate interacts with a basic cluster of amino acids (Lys63, Arg68, and Arg69) and mimics the phosphate ion in the phosphorylated substrate. In the Pin1 crystal structure (Ranganathan et al., 1997), a cluster of hydrophobic amino acids interact with the cyclic proline side chain (Phe134, Met130, Leu122), whereas the side chains of Cys113, His59, His157, and Ser154 surround the peptidyl-prolyl bond. The interaction

of the WW domain of Pin1 with phosphorylated peptide substrates has been elucidated (Verdecia et al., 2000; Wintjens et al., 2001), with affinity constants for the interaction of the order of 100 μ M as a result. The X-ray crystal structure of the full Pin1 protein bound to a doubly phosphorylated peptide (YpSPTp-SPS) from the C-terminal domain (CTD) of RNA polymerase II was reported (Verdecia et al., 2000). We studied by solution nuclear magnetic resonance (NMR) methods two other biologically relevant monophosphate substrates in complex with the WW domain, one derived from the Cdc25 phosphatase of *Xenopus laevis* and another from the human Tau protein (KVSVVRpTPPKSPS) (Wintjens et al., 2001).

Regarding biological functions, Pin1 was first shown to bind phosphoproteins that are involved in the completion of mitosis, such as the Cdc25c phosphatase and the Polo-like kinase Plx1, and, subsequently, it was shown to be involved in other regulation processes, by affecting substrate activity, protein-protein interactions, dephosphorylation, subcellular location, and turnover (for review, *see* Lu et al., 2002a).

In vitro, Pin1-catalyzed prolyl isomerization generates peptide substrates in the preferred trans conformation that are dephosphorylated by the proline-directed and conformation-specific protein phosphatase 2A (PP2A) (Zhou et al., 2000). A direct effect of Pin1 on the activity of the Cdc25 phosphatase, which is phosphorylated by both Cdc2 and Plx1, has also been reported (Stukenberg and Kirschner, 2001). The link between prolyl cis/trans isomerization, conformational change, and activity modulation of the substrate proteins is, however, not yet completely elucidated. More recently, Pin1 has also been involved in the oncogenesis process because it binds phosphorylated c-Jun (Wulf et al., 2001) and β -catenin (Ryo et al., 2001) and upregulates their transcriptional activity, including the expression of cyclin D1 (Wulf et al., 2001; Ryo et al., 2001). The β -catenin transcriptional activity is stimulated by Pin1 because of an increase in its translocation to the nucleus, as a result of the molecular competition between Pin1 and APC to bind the β -catenin to the adenomatous polyposis coli (APC) site (Ryo et al., 2001). Finally, Pin1 binds hyperphosphorylated Tau, characteristic of pathological paired helical filaments in neurofibrillary tangles (Lu et al., 1999b). Pin1 may restore the tubulin polymerization function of the hyperphosphorylated Tau (Lu et al., 1999b). Tau contains about 17 Thr/Ser-Pro motifs, all of which can be phosphorylated in the cellular context (Buée et al., 2000). Amazingly, how-

ever, only the Tau pThr231-Pro232 motif was found to be recognized by Pin1, with a high affinity of 40 nM (Lu et al., 1999b).

Evidence that Pin1 is a key modulator in different cellular processes that could provide a link among the two major pathological processes leading to the formation of amyloid deposits and neurofibrillary tangles in AD can be resumed as follows.

First, Pin1 binds to phospho-Thr231 on Tau proteins and, hence, is found within degenerating neurons, where it is associated to the large amounts of abnormally phosphorylated Tau proteins aggregated into filaments. This abnormal Tau phosphorylation may ultimately be caused by reactivating the cell cycle. Conversely, Pin1 may restore the tubulin polymerization function of these hyperphosphorylated Tau before they aggregate into filaments in Alzheimer neuronal tangles (Lu et al., 1999b).

Second, other proteins involved in AD may bind to Pin1. For instance, the phosphorylated APP possess a potential binding site in its carboxy-terminal domain on residue Thr668, as a conformation-dependent antibody presents a dual specificity for the Thr231 of Tau and the Thr668 of APP. The conformation of these epitopes could represent a characteristic of Pin1 binding specificity (Davies, unpublished data).

Although the literature on Pin1 function is now extensive and shows that Pin1 is involved in numerous regulating processes involving phosphorylation of key proteins (for reviews, see Zhou et al., 1999; Lu et al., 2002a), only one article points to a potential important role of Pin1 in AD disease development (Lu et al., 1999b). In the present work, we investigated, both at the cellular and molecular levels, the role of Pin1 in AD through the study of its interactions with phosphorylated Tau proteins. We also showed that in neuronal cells, Pin1 upregulates the expression of cyclin D1. This, in turn, could facilitate the transition from the G0 phase to the G1 phase in a neuron and, subsequently, neuronal dedifferentiation and apoptosis (Sherr and Roberts, 1999; Nagy, 2000; Liu and Greene, 2001). It allows for the development of new therapeutic strategies.

Materials and Methods

Patients

All patients were previously described in the work of Delacourte et al. (1999) and Sergeant et al. (2002). Control cases were those included in stages 0–3, mild AD (mAD) in stages 6 and 7, and AD in stages

8–10 (Delacourte et al., 1999). One control case was 26 yr old. Other nondemented control cases were age matched for Alzheimer patients.

Brain Tissue Samples

All brain samples (Brodmann area 20) were homogenized in Laemmli sample buffer as described in Sergeant et al. (2002).

Production and Purification of Recombinant Pin1 Proteins

Recombinant Pin1 proteins were produced using Pin1 cDNA in a pET28 vector (Novagen) (a gift of Professor M. Yaffe) in BL21(DE3) Star (Invitrogen SARL) after isopropyl β -D-thiogalactoside (IPTG) induction. His-tagged recombinant Pin1 proteins were purified using the TALON™ metal affinity resin (Clontech).

Antibodies

Anti-Tau and Cyclin D1 Antibodies

Numbering of the Tau epitopes is given according to the longest human 441 Tau isoform. Phosphorylation-dependent monoclonal antibodies included AD2 directed against phosphorylated Ser396–404 and AT180 (Innogenetics, Belgium) labeling phosphorylated Thr231. They recognize phosphorylated Ser/Thr-Pro sites (for review, see Buée et al., 2000). M19G is a well-characterized antiserum, directed against the first 19 amino acids of the tau sequence encoded by exon 1 (Delobel et al., 2002) that recognizes its epitope independently of the tau phosphorylation state. Cyclin D1 monoclonal antibody (HD11) was purchased from Santa Cruz Biotech.

Anti-Pin1 Antibodies

Anti-Pin1 antibodies were affinity-purified goat polyclonal C20, A20, and N19 (Santa Cruz Biotechnology) and protein A purified rabbit IgG (Upstate Biotech).

Production of Anti-Pin1 Antisera

Two New Zealand rabbits were also immunized by purified His-tagged recombinant Pin1 protein and allowed for the obtention of two immune sera against Pin1. Antisera were characterized using both recombinant human Pin1 proteins, lysate of cell transfected with human *Pin1* cDNA and human brain homogenates. Both antibodies recognize a band with an apparent M_r of 18 kDa that was also labeled with other commercially available antibodies (Santa Cruz Biotech. and Upstate Biotech).

Cell Culture and Transfections

SY5Y human neuroblastoma cells were grown in 25-cm² flasks in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM nonessential amino acids and penicillin/streptomycin (Invitrogen) in a 5% CO₂ humidified incubator at 37°C. The isoform of *tau* cDNA with three microtubule-binding domains and the exon 2 sequence (2+3–10–) cloned in pSG5 vector (Stratagene) was a kind gift of Dr. Michel Goedert (Cambridge, UK). It was subcloned into stably eucaryotic expression vector pcDNA3.1Neo (Invitrogen), allowing for a G418 (Invitrogen) selection of stable clones. Transfection was performed into SY5Y cells using the ethyleneimine polymer ExGen500 (Euromedex, France) according to manufacturer's instructions. Clones with high Tau expression were used in the present study (Mailliot et al., 2000).

SY5Y neuroblastoma cells were also used as the basis for the T-Rex system (Invitrogen) allowing inducible Pin1 overexpression. Pin1 cDNA was obtained from Peter Davies (AECOM, Bronx, NY, USA) and subcloned in pcDNA4TO (Invitrogen). The T-Rex system is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *Escherichia coli* Tn10-encoded tetracycline (Tet) resistance operon. Tetracycline regulation in the T-Rex system is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of human Pin1 cDNA.

Differentiation of all cell lines generated was induced with either a 10⁻⁶ M retinoic acid (RA, Sigma) or 10-ng/mL NGF 2.5s (Sigma) treatment in serum free medium for at least 8 d.

Cells were harvested in EDTA and lysates were performed by sonication in radioimmunoprecipitation (RIPA)-modified buffer with the cocktail of protease inhibitor Complete™ (Roche) and the phosphatase inhibitor okadaic acid (Sigma Aldrich).

His-Tagged Pin1 Pull-Down

Tau stably transfected SY5Y cells were lysed in 50 mM Tris-HCl (pH 8), 200 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitors (Complete, Roche). To detect Pin1–Tau protein interactions, SY5Y cell lysates were incubated with TALON resin saturated with His-tagged human Pin1. Bound proteins were extensively washed. All subsequent analyses were performed by immunoblotting.

Electrophoresis, Immunoblotting, and Quantification

Electrophoresis and immunoblotting were performed as previously described (Delobel et al., 2002; Sergeant et al., 2002). In brief, samples were loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Two vertical minigel systems were used: Amersham Biotech and BioRad Protean III (10 or 15% SDS-PAGE). After transfer to nitrocellulose (Hybond, Amersham Biotech), membranes were blocked with 5% skim milk and incubated with primary antibody. Horseradish peroxidase-conjugated antibody was used as a secondary antibody, and the reaction product was detected using the Amersham ECL western blotting system. Films were digitized using an Umax scanner calibrated for optical densities. The IMAGE-MASTER 1D ELITE software (Amersham Biotech) was used to quantify the signal, and data were collected using Excel software (Microsoft).

Production and Purification of Recombinant Pin1 Proteins for Structural Studies

We used Pin1 cloned in pET28 under the control of a T7 promoter for recombinant expression of full-length Pin1 in N-terminal fusion with a 6-Histidines tag in *E. coli*. Recombinant expression of the WW domain with a N-terminal 6-Histidine tag was obtained using a DNA construct derived from the Pin1-PET28 by site-directed mutagenesis using Quick Change (Stratagene), which allowed the introduction of a stop codon mutation in the coding sequence. BL21(DE3) Star *E. coli* strains transformed by the Pin1-PET28 or WW-PET28 plasmid were used to produce 6-Histidine tag Pin1 and WW domain. For uniform labeling of Pin1, the cells were grown at 37°C in Luria-Bertani (LB) medium to an A₆₀₀ nm of 0.6. and then switched in M9 medium (Sambrook et al., 1989) at 30°C. Expression of Pin1 was then induced by addition of 0.4 mM IPTG and the culture was pursued for 3 h. The carbon source was replaced in M9 medium by [¹³C] glucose (Cambridge Isotopes Laboratories, Cambridge, MA) and/or the nitrogen source by [¹⁵N] ammonium chloride (Cambridge Isotopes Laboratories). The 6-Histidine tag proteins were purified on metal-chelating Sepharose fast flow loaded with nickel (Amersham Biotech).

Peptide Synthesis

The Pin1 WW domain was obtained by peptide synthesis using the Boc-benzyl strategy and the HBTU (2-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate) activation

protocol on an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, CA). The tau phosphopeptides were also supplied by peptide synthesis using the Fmoc strategy and activation by HBTU in a 431A peptide synthesizer (Applied Biosystems). After lyophilization the crude peptides were purified by RP-high-performance liquid chromatography (HPLC) on a nucleosil C₁₈ (Macherey-Nagel, Duren, Germany) column using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Homogeneous fractions, as checked by reverse phase on a X-terra RP18 (Waters, Milford, MA) and mass spectrometry on a Quattro II electrospray mass spectrometer (Micromass, Manchester, UK), were pooled and lyophilized.

The sequence of the tau peptides are AcKVSVVRpTPPKSPS for the phospho-Thr231 peptide and AcKVSVVREPPKSPS for the glutamate mimetic peptide.

NMR Spectroscopy

The NMR experiments were performed in a buffer of 50 mM deuterated Tris-HCl, pH 6.3 (Cambridge Isotope Laboratories); 100 mM NaCl, and 5 mM DTT. The spectra were recorded at 20 °C on a Bruker 600-MHz DMX spectrometer (Bruker, Karlsruhe, Germany). Sequential backbone resonance assignment of the WW domain was achieved using the following pairs of triple-resonance (three dimensional) experiments: HNCA/HN(CO)CA and HNCO/HN(CA)CO. Increasing amounts of unlabeled synthetic peptide were added to a [¹⁵N]-labeled WW sample. ¹H-¹⁵N HSQC (heteronuclear single quantum coherence) spectra were recorded at each titration point.

Fluorescence Spectroscopy

Fluorescence spectra were recorded on a PTI (Photon Technology International, New Jersey, USA) fluorescence spectrometer, in the same buffer conditions as for the NMR experiments. Typically, 20 μM WW solutions were titrated with increasing amounts of pThr231 Tau peptide. Excitation was at 280 nm, and the emission spectrum was scanned up to 450 nm.

Results

Pin1 in Alzheimer's Disease

As previously described (Lu et al., 1999b), Pin1 was found in neurofibrillary tangles and degenerating neurites from AD patients (data not shown). No overexpression of Pin1 was observed in AD. Conversely, by immunoblotting, Pin1 expression was slightly decreased in temporal cortex brain

homogenates from AD patients (Fig. 1). However, it should be noted that Pin1 expression was highly heterogeneous among samples.

Pin1 Is Expressed in Neurons

When SY5Y neuroblastoma cells were differentiated into a neuronlike cell by either retinoic acid or NGF for 14 d (Fig. 2A), Pin1 was detected in cell lysates. It exhibited the same *M_r* as Pin1 found in human brain homogenates (Fig. 2B). These data indicated that Pin1 is expressed in human neurons. However, the presence of Pin1 in the neuron is usually considered as a reactivation of the cell cycle. From our experiments and those of Lu et al. (1999b), Pin1 has to be considered a normal neuronal component.

Pin1-Tau Interactions

We confirmed that Pin1 binds to phosphorylated Tau proteins. We previously showed that transfected Tau proteins from SY5Y neuroblastoma cells overexpressing the human Tau isoform 2+3-10- are hyperphosphorylated (Mailliot et al., 2000; Delobel et al., 2002). Here, we demonstrated that these hyperphosphorylated Tau variants bind to Pin1 (Fig. 3). Furthermore, as shown by immunoblotting, the phosphorylated Tau isoform that was bound to Pin1 was immunoreactive for the AT180 antibody that recognizes the phospho-Thr231 Tau epitope (Fig. 3). These data are consistent with those of Lu et al. (1999b), which showed that Pin1 binds to phospho-Thr231.

Structural Data

In order to gain insight in the solution structural properties and interactions between Pin1 and the tau protein, we first solved by NMR the structure of a synthetic Pin1 WW domain (Wintjens et al., 2001). Folding of the synthetic polypeptide proceeded without any problem, identifying this domain as one of the smallest autonomously folding β-sheets known. The structure was close to previously determined X-ray structures and confirmed that the WW domain behaves as an autonomous module within the complete protein. We extended our structural study to the complex with two phosphorylated peptide ligands, one coming from Cdc25 and one coming from Tau. The ¹H resonances of Pin1 WW domain have been deposited in the BioMagResBank (available on the web) under BMRB accession number 4882. The coordinates of the 10 best conformers are deposited in the Rutgers Protein Data Bank under accession code 1I8H for the complex phospho-Thr231 Tau peptide/WW domain (Wintjens et al., 2001). In both complexes, the Pin1 WW domain binds its peptide ligand

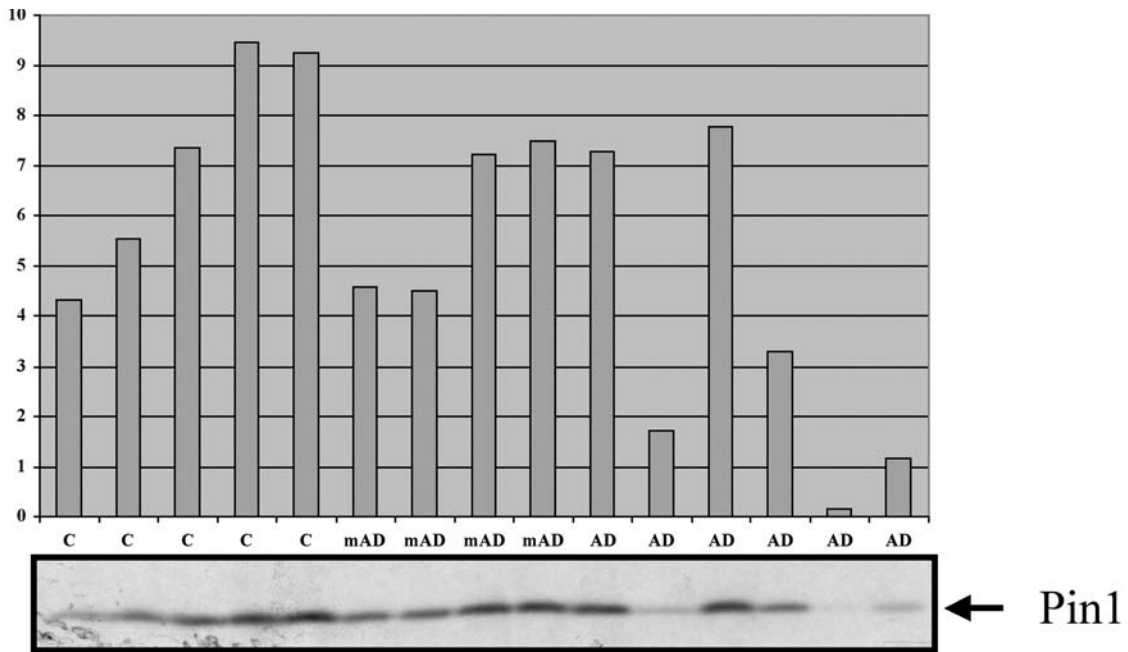


Fig. 1. Pin1 expression in Brodmann area 20 (temporal cortex) in nondemented control cases (C), mild AD patients (mAD), and AD patients (AD). Pin1 is detected at 18 kDa. Quantitative analysis of Pin1 immunoreactivity among samples indicates that values are highly heterogeneous, but there is a slight decrease in AD patients. All data are expressed in arbitrary units.

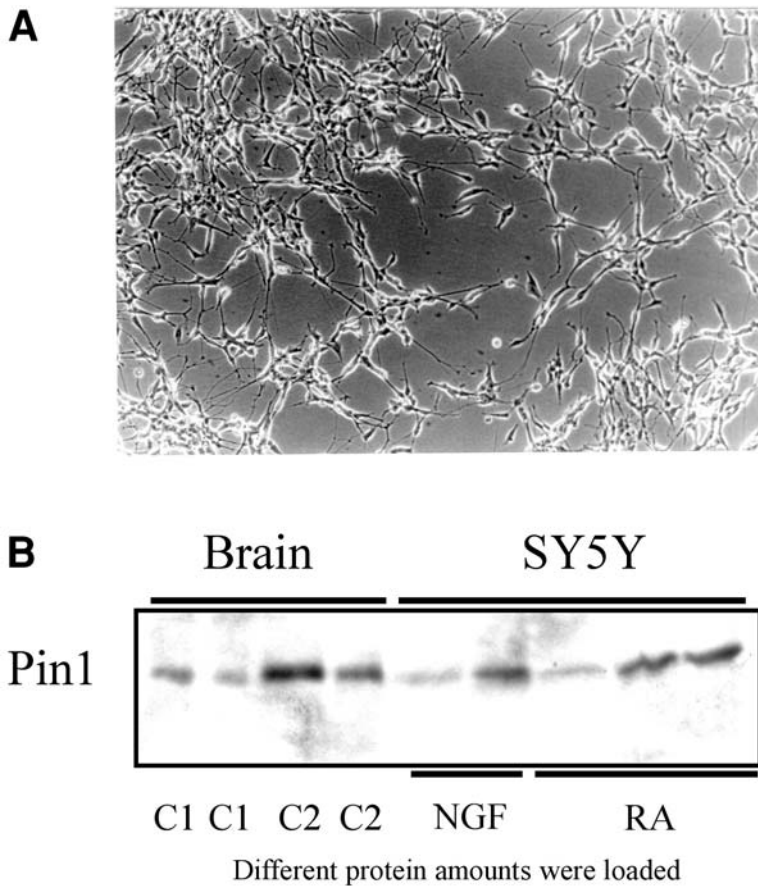


Fig. 2. Pin1 expression in NGF- and RA-differentiated neuroblastoma SY5Y cells. (A) Neuroblastoma SY5Y cells after 7 d NGF; (B) Pin1 immunoblotting in brain homogenates and differentiated SY5Y cells. No difference in molecular weight was observed among samples. Different volumes were loaded on gels.

in the same direction as found in the X-ray structure of the Pin1 protein complexed to a phosphopeptide derived from the CTD of the polymerase (Verdecia et al., 2000). Moreover, our structural data clearly dissect the interaction in two major contributions: The phosphate group strongly interacts with the side chain of the arginine 12 residue in the β 1- β 2 turn of Pin1WW, whereas the proline moiety inserts in the aromatic groove formed by the Trp and Tyr of the WW domain (Fig. 4). A further element, crucial for the functional interpretation of our structural data, is that the phospho-Tau peptide interacts with the Pro232 in a trans conformation. Both modeling results on the CTD peptide–Pin1 complex (Verdecia et al., 2000) and our data with the Cdc25 peptide, where a significant fraction of the molecules is characterized by a proline in the cis configuration (Wintjens et al., 2001) demonstrate that the WW domain cannot accommodate a phospho-Thr-Pro moiety with the latter residue in the cis configuration.

Interaction Between Pin1 WW Domain and Tau

Among the different potential phosphorylation sites on Tau, only the phosphorylated Thr231 was reported to be crucial for Pin1 interaction, and a high-affinity binding was reported. Our initial titration results with the synthetic domain confirmed this interaction, but found a much weaker interaction characterized by a dissociation constant K_d of the order of 300 μ M. Because this difference might be of true biological importance, we further investigated the interaction both by NMR spectroscopy and fluorescence. Several differences between our interaction study and the initial data of Lu et al. (1999b) can be pinpointed: we used a solution technique whereas the initial data were obtained by enzyme-linked immunosorbent assay (ELISA), which is a surface technique. Second, the protein itself was different: We used a synthetic WW domain, in contrast to the entire Pin1 protein used in the ELISA study. Finally, the peptide sequence reported was not exactly the one of Tau, but differed in one residue (S227A). In order to eliminate a potential cooperativity between the WW and catalytic domain as the origin of the discrepancy, we repeated the NMR experiments with 15 N-labeled recombinant proteins encoding the WW domain or the full-length Pin1 protein. The labeling has the main advantage that the proton resonances can be spread out in a second frequency dimension, yielding a much clearer spectrum. Titration of the phosphorylated ligand affects those amide resonances that are close in space to the ligand (Fig. 5), and fitting the chemical-shift variations as a func-

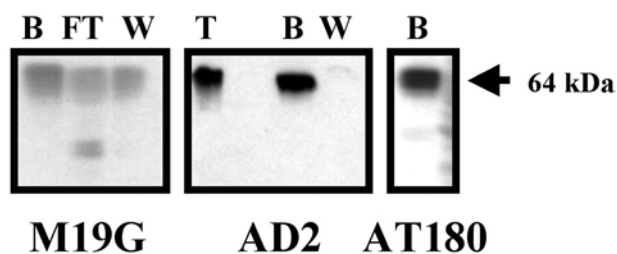


Fig. 3. Interaction of Pin1 with phospho-Tau from Tau-transfected SY5Y cells. Beads containing His-tagged Pin1 were incubated with lysates of SY5Y cells overexpressing the 2+3–10– Tau isoform (T, total cell lysate). Three fractions were obtained: FT, unbound materials; W, wash fractions; B, bound materials. The different fractions were then analyzed by immunoblotting. Tau proteins were detected with either the anti-Tau polyclonal antibody (M19G) or phosphorylation-dependent monoclonal antibodies (AD2, anti-phospho-S396 and 404 and AT180, anti-phospho-T231). Even if Tau proteins are found in all fractions (M19G-B, FT, and W), only the phosphorylated Tau proteins bind to Pin1 (AD2 and AT180-B).

tion of ligand concentration leads to a value of the dissociation constant. Using both the isolated WW domain and the full-length Pin1 protein, we found resulting K_d values of 300 μ M for both systems, excluding any cooperativity between the WW and catalytic domains. As the WW domain contains one Trp residue in the binding pocket (Fig. 4), we further examined the use of fluorescence spectroscopy to characterize the interaction. The fluorescence of one Trp side chain was indeed enhanced (and slightly blue shifted) upon interaction with the phosphopeptide, and titration with the ligand was therefore straightforward (Fig. 6). This independent measurement confirmed the data obtained by NMR spectroscopy, leading us to the conclusion that the K_d value in solution is of the order of 200–300 μ M. We are currently investigating by a different surface technique, based on surface plasmon resonance, whether we can understand the discrepancy between our results and those previously reported in the literature and whether the one or the other value is more representative for the interaction between Pin1 and the Tau protein in the PHF tangles.

Pin1 and Cyclin D1 Regulation

Pin1 upregulates the expression of cyclin D1 through c-jun activation and stabilization of β -catenin (Ryo et al., 2001; Wulf et al., 2001; You et al., 2002). However, there is no evidence that such regulation of cyclin D1 is also observed in a neuronal cell. To ascertain that cyclin D1 expression is also

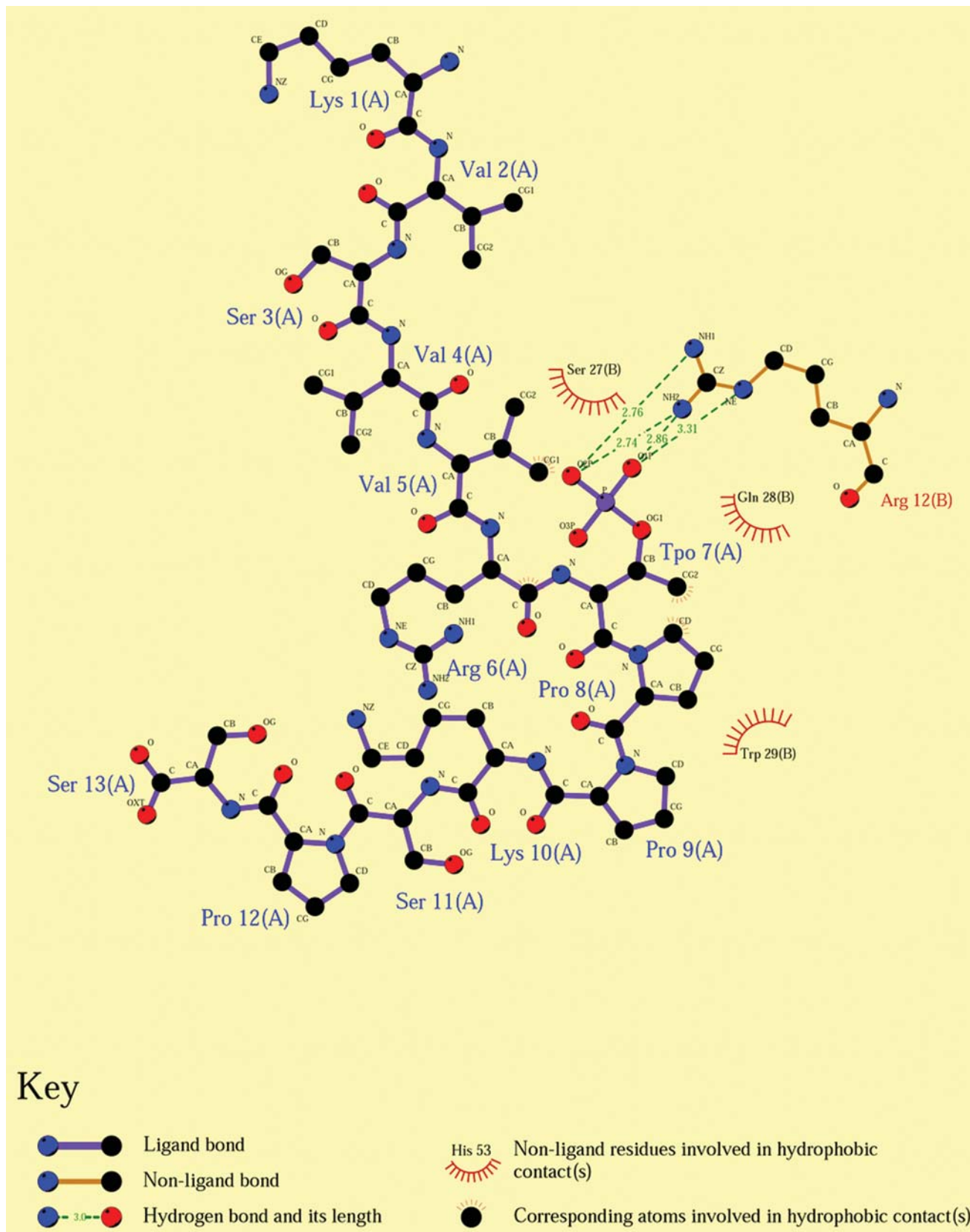


Fig. 4. Two-dimensional plot highlighting the interactions between the Pin1 WW domain and a phospho-Thr231 peptide from Tau.

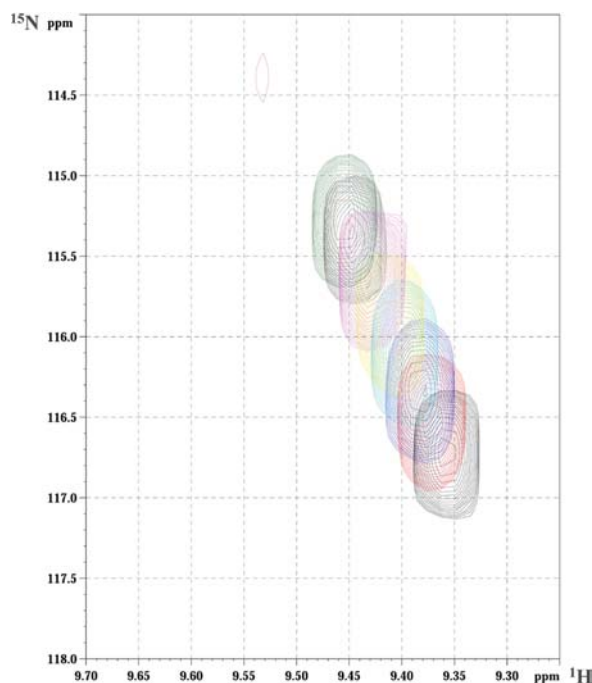


Fig. 5. Interaction of WW with the phospho-Thr231 peptide. Overlaid sections of ^{15}N -HSQC spectra acquired on a $300\text{-}\mu\text{M}$ ^{15}N -labeled WW domain sample, before (in black contours) and after addition of 0.35 (red), 0.70 (dark blue), 1.35 (light blue), 2.00 (yellow), 3.35 (pink), 6.70 (gray), and 13.30 (green) molar equivalent of phospho-Thr231 peptide. A gradual chemical-shift perturbation of residue Gln28 can be observed, as a result of fast exchange between the bound and unbound forms of the WW domain on the NMR time scale.

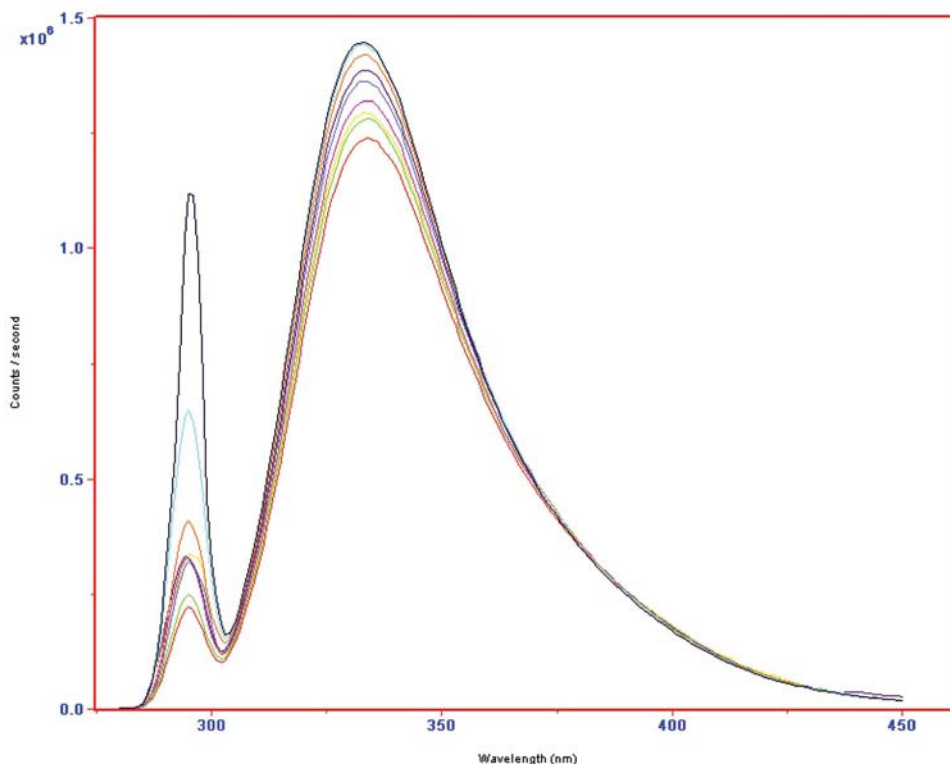


Fig. 6. Fluorescence spectra of a $20\text{-}\mu\text{M}$ solution of the WW domain with increasing amounts of phospho-Thr231 peptide. The values of the peptide:WW domain molar ratio range from 0:1 to 40:1, corresponding to fluorescence curves going from red to black.

controlled by Pin1 in neuronal cells, we used either inducible overexpression of Pin1 or the inhibition of the isomerase Pin1 activity by juglone.

In Pin1-inducible neuroblastoma SY5Y cells, we found a strong correlation between Pin1 overexpression and an increase in cyclin D1 immunoreactivity (Fig. 7A). Forty-eight hours after Pin1 induction, an increase of cyclin D1 immunoreactivity was observed by immunoblotting. To further support these observations, we used the Pin1 inhibitor juglone to inhibit the isomerase activity. Cyclin D1 immunoreactivity was highly decreased in SY5Y cells treated by juglone. Altogether, these data clearly demonstrated that Pin1 regulates cyclin D1 expression and/or stability.

Discussion

Pin1, A Therapeutic Target in AD?

The results from our immunoblot experiments demonstrated that Pin1 is a protein normally expressed in neurons. Moreover, we also confirmed that Pin1 is a component of NFTs and slightly decreased in AD. However, this decrease of PIN1 protein level in AD brain homogenates may not be the most interesting point. Posttranslational modifications of PIN1, such as phosphorylation, could prove to be different between AD and control cases. It would be of great interest because phosphorylation of Pin1 at Ser16 precludes the binding of PIN1 WW domain to its phosphorylated substrates (Lu et al., 2002b). We indeed detected phosphorylated Pin1 variants in AD brain samples (data not shown), suggesting that Pin1 function could be regulated in the disease by a combination of downregulation of expression level and posttranslational modification.

In the present work, we confirmed a direct physical interaction between Pin1 and the phosphorylated Tau proteins, in agreement with the localization of Pin1 in the NFTs observed by Lu et al. (1999b).

However, the role of the Pin1-tau interaction in the disease development is not clear, as Pin1 could have conflicting effects. On one hand, Pin1 becomes depleted from the nucleus within diseased neurons when it is redirected to the large amounts of abnormally phosphorylated Tau proteins (Lu et al., 1999b). This depletion from the nucleus may ultimately contribute to neuronal cell death by reactivating the cell cycle leading to apoptosis. On the other hand, it was proposed that Pin1 could restore the ability of phosphorylated Tau proteins to bind microtubules and promote their assembly *in vitro*. Proline isomerization may indeed facilitate Ser/Thr dephosphoryla-

tion by phosphatase 2A (Zhou et al., 2000). Both hypotheses, however, implicated that Pin1 is involved in the neuronal fate, after Tau is abnormally phosphorylated.

However, there is much evidence that a reactivation of the cell cycle may be an early event in neurodegeneration and trigger neuronal death (Nagy, 2000; Delobel et al., 2002). We propose here a new hypothesis for an earlier involvement of Pin1 in neurodegeneration in tauopathies. We demonstrated that it is possible to increase levels of cyclin D1 by inducing Pin1 in neurons. Conversely, we showed that there was a drop of cyclin D expression after cell treatment by juglone, a Pin1 inhibitor. This is in agreement with other cellular models used to show that Pin1 upregulates the expression of cyclin D1 through c-jun activation and stabilization of β -catenin (Ryo et al., 2001; Wulf et al., 2001; You et al., 2002). Upregulation of cyclin D1 in a neuron could facilitate the transition from the G0 phase to the G1 phase and subsequently neuronal dedifferentiation and apoptosis (Sherr and Roberts, 1999; Nagy, 2000; Liu and Greene, 2001). Because Pin1 seems a key actor in this early event of cell cycle reactivation, it could be a good candidate for new therapeutic strategies.

The cellular models that are here presented could be valuable tools to evaluate *in vivo* the efficiency of new drugs, affecting the neuron fate by the intermediate of PIN1 regulation. Effects of these inhibitors could be easily quantified by measuring either cyclin D1 mRNA or protein levels.

In a first step, however, a large amount of molecules should be designed and screened *in vitro*. This requires an understanding of Pin1-phospho-Tau interactions at the molecular level and the setting up of an assay that would allow screening for inhibition.

Structure and Interactions

Our NMR model of the Pin1 WW domain complexed with a phosphorylated Tau peptide centered around Thr231 clarifies the molecular basis of the Pin1-Tau interaction, but equally raises a number of intriguing questions. First, it is clear from our results and from the X-ray structure of Pin1 complexed to a peptide derived from the C-terminal domain of polymerase II (Verdecia et al., 2000) that the interaction is mainly limited to the phosphorylated side chain of the threonine residue and the proline stacking between the aromatic clamp formed by Trp29 and Tyr18 side chains on the WW domain (Fig. 4). The former is the most important, and cannot readily be replaced by a glutamate moiety (Smet, unpub-

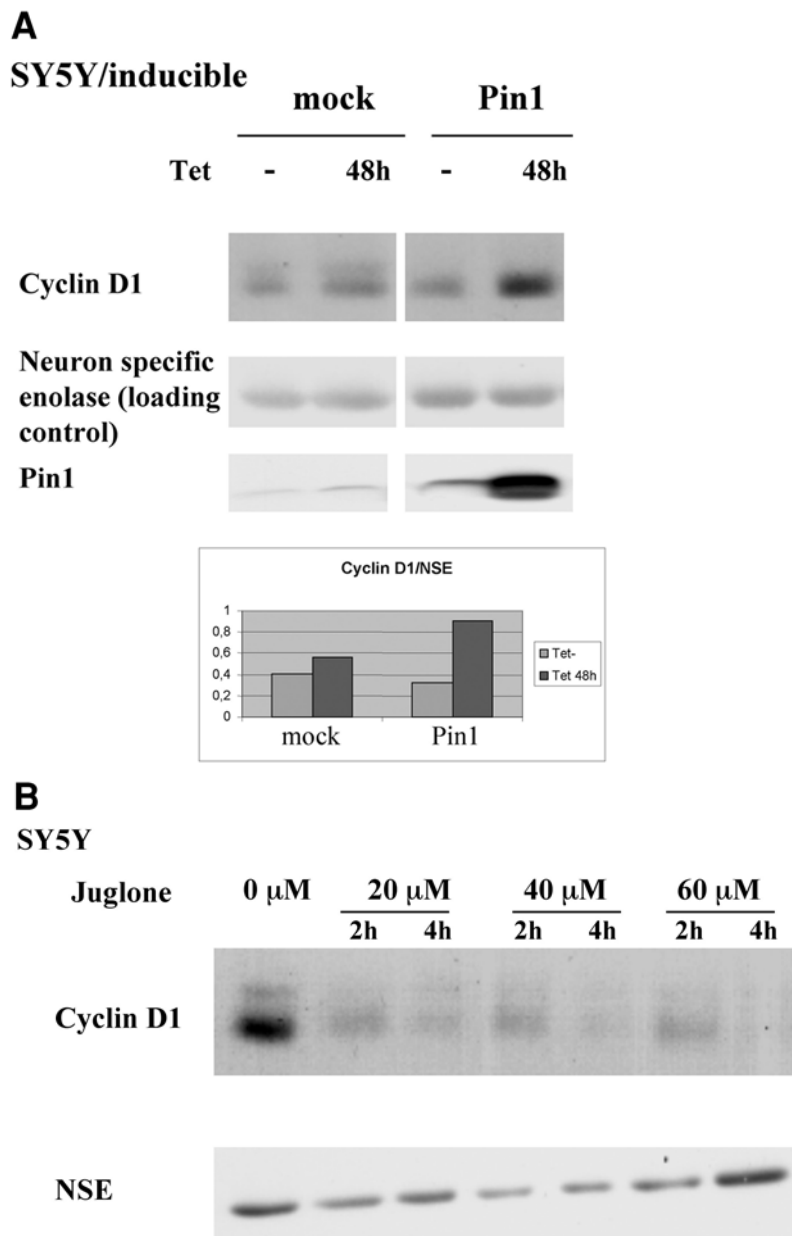


Fig. 7. Cyclin D1 immunoblotting and Pin1 expression and/or activity. (A) Analysis of cyclin D1 expression in Pin1-inducible SY5Y neuroblastoma cells. The increase in cyclin D1 immunoreactivity is correlated to Pin1 over-expression. (B) Analysis of cyclin D1 expression in juglone-treated SY5Y neuroblastoma cells. The inhibition of rotamase activity by juglone leads to a decrease in cyclin D1 immunoreactivity.

lished data). This latter factor will form a serious obstacle to the study of a larger fragment of Tau and its interaction with Pin1, as the side-specific phosphorylation can probably not be mimicked by a Thr231Glu mutation. However, the true question raised by the molecular description is that of the specificity. Why would Pin1 interact only with the pThr231-Pro moiety and not with the other phosphopeptides that contain exactly the same dipep-

ptide, if the other residues do not directly interact? Of course, the peptide derived is in the proline-rich region of Tau, and therefore, one cannot exclude that entropic factors related to the lesser flexibility of the proline residue contribute significantly to the binding affinity. A second question is the affinity of Pin1 for its substrate. If the WW domain behaves as a targeting module, bringing the catalytical domain close to its substrate, a nanomolar affinity would lead to

an almost permanent complex between Pin1 and its substrate molecule. If Pin1 is to fulfill its catalytical role on many substrate proteins, the high affinity and subsequent permanent binding character do not seem highly efficient. Finally, of crucial importance is the exclusive interaction between the WW domain and the trans conformation of the proline in the interaction motif. The catalytical activity of Pin1, indeed, is the prolyl cis/trans isomerization of the proline in the interaction motif. This activity was demonstrated both in an indirect assay based on the trans-selective enzymatic cleavage by chymotrypsin and by a direct assay based on our NMR spectroscopy, in the case of the *Arabidopsis thaliana* Pin1 protein (Landrieu et al., 2000). Importantly, the same trans selectivity was shown for PP2A, a phosphatase equally implicated in regulation of the cell cycle and of Tau phosphorylation (Zhou et al., 2000). Therefore, one possible hypothesis about the molecular function of the WW domain is that it first binds to a phosphorylated site with the proline in the trans conformation (Thr231?), and thereby increases locally the concentration of the catalytic Pin1 domain. This latter can then perform its catalytical activity on a different phosphoThr-Pro moiety. Alternatively, the Pin1 catalytical domain could first change the conformation of a proline from cis to trans, whereby the latter conformation would be stabilized by the WW domain for a sufficient period of time in order for other molecular partners such as PP2A to come in. Which of the possible scenarios is valid in vitro and in vivo remains to be determined.

Evidence that the catalytical activity of Pin1 promotes a conformational change in its substrate was most pronounced for Cdc25 (Stukenberg and Kirschner, 2001), but an equivalent scenario might well be valid for Tau. It remains intriguing how the local conformational change can be amplified into a large-scale reorganization of the substrate proteins, being that the proline residues are invariably located in a highly flexible and unstructured part both for Tau and Cdc25. However, only very little is known about the structure of Tau in the tangles found in Alzheimer's diseased brains. Certain antibodies are capable of specific recognition of this diseased form, and it might well be that the relevant structural features of Tau cannot easily be detected based on its form in solution.

The above-described titration results between the WW domain and the phosphoThr231-containing peptide by both NMR spectroscopy and fluorescence can easily be extended to probe the molecular interaction between potential inhibitors and the pro-

tein module. Indeed, a similar approach called "structure-activity research (SAR) by NMR" was already proposed to generate the interaction data for libraries of small molecules stemming from combinatorial chemistry, while maintaining the high throughput mode required by the large number of molecules to be tested (Hajduk et al., 1997). The advantage of this method over the more traditional fluorescence-based methods is that it immediately generates a molecular image of the interaction site, but because of the necessity of labeled protein and the higher quantities of protein, we envision a mixed strategy with a primary screen by fluorescence and only in a secondary phase by NMR spectroscopy. Screening will be performed on the WW domain rather than on the catalytical domain, although a small molecule inhibitor, the juglone, was already described in the case of a parvulin of *E. coli* (Henning et al., 1998). However, juglone acts by forming a covalent bond with a free cysteine residue and subsequently provoking a partial degradation for the protein. Because this interaction is most probably highly unspecific, it cannot be considered a good lead. The catalytic loop of Pin1 equally binds the phosphoThr-Pro dipeptide, but with a much weaker affinity than the WW domain (Landrieu et al., 2002), making this site a less attractive target for rational drug development based on the structural data known.

The results that are presented here contribute to show that PIN1 could be an important drug target, as it is possibly involved in early stages of neurodegeneration. We described the tools that we have developed, to first design, and then assay a large number of active compounds in vitro to block Pin1 WW binding to its neuronal substrates. The promising compounds will then be assayed as potential drugs to block the reactivation of the cell cycle in neurons in the cellular models that we have characterized.

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