A homologue of the human site-specific prolyl cis/trans isomerase PIN1 was identified in Arabidopsis thaliana. The PIN1At gene encodes a protein of 119 amino acids that is 53% identical with the catalytic domain of the human PIN1 parvulin. Steady-state PIN1At mRNA is found in all plant tissues tested. We show by two-dimensional NMR spectroscopy that the PIN1At is a prolyl cis/trans isomerase with specificity for phosphoserine-proline bonds. PIN1At is the first example of an eukaryotic parvulin without N- or C-terminal extensions. The N-terminal WW domain of 40 amino acids, typical of all the phosphorylation-dependent eukaryotic parvulins, is absent. However, triple-resonance NMR experiments showed that PIN1At contained a hydrophobic helix similar to the α helix observed in PIN1 that could mediate the protein-protein interactions.

Peptidyl prolyl cis/trans isomerases (PPIases) are enzymes that catalyze the cis/trans isomerization of the peptide bond preceding proline, an intrinsically slow process. PPIases are divided into the following three structurally distinct classes: cyclophilins, FK506-binding proteins, and parvulins. The parvulin class comprises eukaryotic enzymes that were shown to be essential for growth, such as the PIN1 from human (1) or the Saccharomyces cerevisiae ESS1/PPTF1 (2–4). These enzymes preferentially recognize substrates with a phosphorylated serine or threonine N-terminal to the proline residue (5, 6) and interact with a subset of phosphoproteins that are involved in the completion of mitosis, such as the CDC25 phosphatase and the polo-like kinase PLX1 (7, 8). Most recently, the human PIN1 protein was also found to interact with the microtubule-associated protein tau (9). However, classification has become more complex with the isolation of the human Par14 parvulin enzyme that does not depend on substrate phosphorylation (10), much like the bacterial parvulin Par10 (11). For clarity, the phosphorylation-dependent class of eukaryotic parvulin PPIases will be designated PIN-type PPIases. Further members of the PIN-type PPIase class are the DODO from Drosophila melanogaster (12), the PINA from Aspergillus nidulans (7), and the SSP1 from Neurospora crassa (13).

All the PIN-type PPIases have high sequence similarity in the catalytic domain, in particular a cluster of basic amino acids (lysine 63, arginine 68, and arginine 69 in the human PIN1) that are thought to be responsible for the site-specific PPIase activity (5, 6). In addition to the catalytic domain, PIN-type PPIases have a WW domain of 38–40 amino acid residues (14–16) at their N terminus. Two invariant tryptophans and a high content of proline and hydrophobic aromatic residues characterize WW domains. The WW domain of the human PIN1 has recently been shown to bind phosphorylated peptides and mitotic phosphoproteins through interaction with P-Ser and phosphothreonine (17). The WW domain binding activity is required for PIN1 to interact with its substrate in vitro and to perform its essential function in vivo (17).

Here we describe the isolation and characterization of an Arabidopsis thaliana PIN1 homologue. The plant PIN-type PPIase has a characteristic catalytic domain but no N-terminal WW protein-protein-binding domain or comparable module. We demonstrate the in vitro P-Ser-dependent PPIase activity of the PIN1At by using two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy.

**EXPERIMENTAL PROCEDURES**

Cloning of A. thaliana PIN1 Homologue—The human PIN1 amino acid sequence was used to search for homologous proteins in A. thaliana sequence data bases. A 69-amino acid sequence was found that is encoded by the A. thaliana 212-base pair transcribed sequence of the expressed sequence tag clone PAP1864 (accession number F13919). The 212-base pair EcoRI–XhoI cDNA fragment from the PAP1864 clone (Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH) was used as probe to screen an A. thaliana cDNA flower library (Arabidopsis Biological Resource Center). Approximately 100,000 plaque-forming units were screened under high stringency conditions, according to the manufacturer’s procedure (Amersham Pharmacia Biotech). Only one positive hybridizing plaque could be identified. After purification, the positive plasmid was excised in vivo into the PIN4 plasmid, which was then used for sequencing.
at 72 °C. The obtained PCR fragments were separated on a 2% agarose gel and blotted on nylon filters (Hybond-N™; Amersham Pharmacia Biotech). Hybridizations were performed at 65 °C using fluorescein-labeled PIN1At and ACT2 probes synthesized with the Gene Images™ random-prime labeling module (Amersham). The signals were detected with the Imagequant™ CDF-System detection module (Amersham Pharmacia Biotech). The same results were obtained with 15, 20, and 25 cycles, demonstrating that in the conditions used none of the reaction components were limiting.

Expression of A. thaliana PIN1At Gene in Escherichia coli—The PIN1At coding region was amplified from the PIN4 plasmid by PCR with the primers 5′-CTGACCAATGCTGTG-3′ (Stratagene, La Jolla, CA). The PCR product was subcloned into the NdeI and XhoI cloning sites of pET19b (Novagen, Madison, WI), to obtain PIN1At-pET19b. The PIN1At gene is located downstream of a T7lac promoter, in frame with a sequence encoding a 10-histidine tag followed by an enterokinase recognition site. Escherichia coli BL21(DE3) cells (Novagen) containing the PIN1At-pET19b plasmid were grown at 37 °C in M9 medium (18), supplemented with 100 μg/ml of ampicillin, to obtain a cell density corresponding to an A595 of 0.6. Then expression of the PIN1At gene was induced by addition of 0.4 mM isopropyl-β-D-thiogalactoside, and culture was continued for 4 h at 30 °C.

Purification of Recombinant PIN1At—Cells were collected in lysis buffer containing 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM EDTA using a DMX 600-MHz spectrometer (Bruker) with a triple-resonance probe head containing the peptide were lyophilized. The AcWFYS/PO/H2PRLR-NH2 peptide was synthesized starting from Rink amide resin (0.58 mmol g−1) using the Fmoc strategy and activation by HBTU and HOBT in a CD6 spectrophotometer (Jobin Yvon, Longjumeau, France). The sample was 43 μM PIN1At in 50 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF. Spectra were recorded from 180 to 250 nm using a 0.1- and 1-mm path length cell at 20 °C. Backbone carbon and hydrogen resonances were assigned from a set of triple resonance NMR experiments (19) recorded on a 1-mM 13C,15N-labeled backbone complex points in the acquisition domain, 32 scans per increment, 256 points were added to the samples to provide a reference. Experimental parameters as follows: recycle delay of 1 s, spectral width of 10 ppm, 1,024 fill in both dimensions.

Peptide Synthesis—The AcWFYS/PO/H2PRLR-NH2 peptide was synthesized starting from Rink amide resin (0.58 mmol g−1) using the Fmoc strategy and activation by HBTU and HOBT in a CD6 spectrophotometer (Jobin Yvon, Longjumeau, France). The sample was 43 μM PIN1At in 50 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF. Spectra were recorded from 180 to 250 nm using a 0.1- and 1-mm path length cell at 20 °C. Backbone carbon and hydrogen resonances were assigned from a set of triple resonance NMR experiments (19) recorded on a 1-mM 13C,15N-labeled PIN1At sample in 50 mM deuterated Tris-HCl, pH 7.0 (Cambridge Isotope Laboratories, Cambridge, MA), 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF. The cleavage with enterokinase left one extra histidine residue at the N terminus.

Secondary Structure Analysis—The circular dichroism spectra were recorded on a CD6 spectrophotometer (Jobin Yvon, Longjumeau, France). The sample was 43 μM PIN1At in 50 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF. Spectra were recorded from 180 to 250 nm using a 0.1- and 1-mm path length cell at 20 °C. Backbone carbon and hydrogen resonances were assigned from a set of triple resonance NMR experiments (19) recorded on a 1-mM 13C,15N-labeled PIN1At sample in 50 mM deuterated Tris-HCl, pH 7.0 (Cambridge Isotope Laboratories, Cambridge, MA), 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF. The cleavage with enterokinase left one extra histidine residue at the N terminus.

RESULTS

Cloning of PIN1At—The PIN1At gene was cloned during a project involving the isolation of cell cycle-related genes in A. thaliana. The cloning strategy combined data base searches with the PIN1 human sequence and A. thaliana cDNA library screens with a partial cDNA sequence, obtained from the Arabidopsis Biological Resource Center, as probe. The isolated PIN1At cDNA comprised a coding sequence of 357 base pairs that encoded a 119-amino acid protein with a molecular mass of 13018 Da and a calculated pI of 9.9. The genomic sequence of PIN1At (this work) is from A. thaliana (accession number AAD20122), PIN1 is from human (AAC50492), DODO is from D. melanogaster (AAC28408), PINA is from N. crassa (AAC49884), SSP1 is from N. crassa (CAAA6818), SPC162403 is from Schizosaccharomyces pombe (CAAA20423), ESS1/PTF1 is from S. cerevisiae (S52764/CAAA95961), and Par10 is from E. coli (S48658). Asterisks indicate the potential anion-binding sites, and secondary structure elements of the human PIN1 are indicated above the alignment (6).

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complex points in the indirect dimension, and phase discrimination by the States-Haberkorn method (20). Water presaturation was obtained by low power irradiation at the water frequency. To the 500-μl peptide samples, 5-10 μl of the 1 mM stock solution of PIN1At protein were added to a final concentration of 10 or 20 μM. EXSY spectra were recorded with mixing times of 50, 100, 200, 300, and 400 ms. Spectra were transferred after squared sine multiplication and one level of zero fill in both dimensions.

FIG. 1. Amino acid sequence alignment of the PIN-type PPIase of the parvulin family using the CLUSTALW program. Residues identical to the column consensus are white on a black background; similar residues to the column consensus are shaded in gray. PIN1At (this work) is from A. thaliana (accession number AAD20122), PIN1 is from human (AAC50492), DODO is from D. melanogaster (AAC28408), PINA is from N. crassa (CAAA6818), SPC162403 is from Schizosaccharomyces pombe (CAAA20423), ESS1/PTF1 is from S. cerevisiae (S52764/CAAA95961), and Par10 is from E. coli (S48658). Asterisks indicate the potential anion-binding sites, and secondary structure elements of the human PIN1 are indicated above the alignment (6).

FIG. 2. Purification of Recombinant PIN1At. The PIN1At protein was solubilized at 1 mg/ml in 50 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF. The cleavage with enterokinase left one extra histidine residue at the N terminus.

FIG. 3. Secondary Structure Analysis of PIN1At. The PIN1At fusion protein was pooled, acidified by adding formic acid, and further fractionated on a 40-m1 Puriflex RPHPLC (Phyto-Pharmacia, Uppsala, Sweden) column (Omnifit, Cambridge, United Kingdom) equilibrated in 0.1% trifluoroacetic acid with an acetonitrile gradient. The PIN1At-containing fractions were lyophilized. The fusion protein was then digested with EnterokinaseMax™ (Invitrogen, Carlsbad, CA) in enterokinase buffer to remove the 10-histidine tag. Reverse phase liquid chromatography was used to remove the residual fusion protein in the same conditions as described above. The PIN1At protein was solubilized at 1 mg/ml in 50 mM deuterated Tris-HCl, pH 7.0 (Cambridge Isotope Laboratories, Cambridge, MA), 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF. The cleavage with enterokinase left one extra histidine residue at the N terminus.

Secondary Structure Analysis—The circular dichroism spectra were recorded on a CD6 spectrophotometer (Jobin Yvon, Longjumeau, France). The sample was 43 μM PIN1At in 50 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF. Spectra were recorded from 180 to 250 nm using a 0.1- and 1-mm path length cell at 20 °C. Backbone...
PPIase Activity—The isolated cDNA was used for recombinant production of PIN1At to validate the presumed PPIase activity of the protein. In the *in vitro* PPIase activity of PIN1At was investigated by 2D-NMR EXSY (23). Previously, this type of 2D spectroscopy had been used as an alternative to the circular dichroism spectrum analysis demonstrated that the recombinant PIN1At used here had a defined conformation in solution and showed that the secondary structure of PIN1At was composed of 27% residues in helical conformation (Fig. 3). A similar α helix content (34%) was predicted from the deviation of the observed Hα, Cα, and CO chemical-shift values of PIN1At from their random coil values (22). Based on the Co data, four predicted helices were located from residues 38 to 56, 59 to 66, 68 to 73, and 87 to 96 (Fig. 4). The longer helix, predicted between residue 38 and 56 matched the length and location of the 20-amino acid scaffolding α helix observed in the PIN1 crystal structure (6). The chemical shift plot also identified a β strand located from residues 6 to 16.

**DISCUSSION**

We report the characterization of an *A. thaliana* PPIase PIN1At that shares a high degree of sequence similarity with the catalytic core of PIN-type PPIases. Globally, PIN1At resembles most closely the prototype *E. coli* Par10 parvulin (11), because they do not contain any N- or C-terminal extensions. All other members of the PIN-type parvulin family have an additional WW domain at their N terminus. Structurally, this small domain is linked to the PPIase domain in human PIN1 by a flexible linker of 8 amino acids in human PIN1 (1, 6) or an additional WW domain at their N terminus. Structurally, this small domain is linked to the PPIase domain in human PIN1 by a flexible linker of 8 amino acids in human PIN1 (1, 6) or an additional WW domain at their N terminus. Structurally, this small domain is linked to the PPIase domain in human PIN1 by a flexible linker of 8 amino acids in human PIN1 (1, 6) or separated by a poly(Q) stretch from its catalytic domain in the *N. crassa* homolog SSP1 (13). In the crystal structure of human PIN1, an interdomain cavity separating the N-terminal WW
domain from the catalytic PPIase domain was observed with scaffolding helix $a_1$ contributing a total of 9 residues to its wall and creating a 23-Å deep internal surface opposite to the hydrophobic pocket of the WW domain (6). Until now, this helical insertion into the core PPIase fold has been found only in the PPIase domains of PIN1, ESS1, DODO, and SSP1, which all possess an N-terminal WW domain. The described PIN1At protein lacks the N-terminal domain but contains a sequence that is highly homologous to the PIN1 $a_1$ helix. Furthermore, preliminary structural data by circular dichroism and conformational $^{13}$C chemical-shift deviations from random coil values are consistent with this stretch being in a helical conformation. Functionally, the role of a protein-protein interaction module was initially recognized for the WW domains (14), but only very recently has it been shown that the PIN1 WW domain interacts specifically with a number of proteins phosphorylated on one or more serine or threonine residues (17). This interaction is essential for its in vivo activity, because neither the N-terminal WW domain nor the C-terminal PPIase domain could replace the essential function of ESS1/PTF1 in yeast. Moreover, all WW mutants of PIN1 that did not bind phospho-proteins failed to support cell growth in this assay, underlining its functional importance. The role of this binding has been proposed to be the processive isomerization of heavily phosphorylated protein substrates of PIN1 by an enhanced recruitment of the PIN1 near its substrates (17). From both the absence of a WW domain and the presence of a hydrophobic $a_1$ helix in PIN1AT, we might speculate that the latter could engage in hydrophobic interactions with other proteins, thereby favoring close proximity between the catalytic domain and its potential substrates. Despite the global resemblance to the E. coli parulin due to the lack of the WW domain, PIN1AT does show specificity for peptide substrates with P-Ser preceding proline, as is the case for all other PIN-type PPIases. Furthermore, a peptide similar to the optimal PIN1 peptide substrate was found to be a good substrate for PIN1AT. Mechanistically, this observation is consistent with the presence of a cluster of basic amino acids in PIN1AT (lysine 15 and arginine 20 and 21), implicated in the phosphate specificity in human PIN1 (5, 6).

**Fig. 5.** Phosphorylation-dependent PPIase activity of PIN1At. The $\delta$ proline proton region of the 2D EXSY spectra of the peptide substrates is shown. Two diagonal peaks at 3.81 and 3.9 ppm and two overlapping diagonal peaks at 3.61 ppm correspond to the $\delta$1/$\delta$2 protons of the proline in cis and trans conformation, respectively. The additional (circled) exchange cross-peaks between proline $\delta$ protons in the presence of PIN1At prove the acceleration of the interconversion between the cis and trans isomers. A, 1 mM Ac-WFYS(PO$_3$H$_2$)PRLR-NH$_2$; B, 1 mM Ac-WFYS(PO$_3$H$_2$)PRLR and 10 $\mu$M PIN1At; C, 2 mM Ac-WFYPRLR-NH$_2$; D, 2 mM Ac-WFYPRLR-NH$_2$ and 20 $\mu$M PIN1At. Mixing time is 300 ms.

**Fig. 6.** Time and concentration dependence of the PIN1At PPIase activity with the peptide Ac-WFYS(PO$_3$H$_2$)PRLR-NH$_2$ as substrate. The integrated volume of the cross-peak between $\delta$ proline protons normalized by the intensity of the corresponding diagonal peak is plotted as function of the mixing time for two concentrations of PIN1At (10 and 20 $\mu$M, triangles and circles, respectively).
This potential anion-binding site could even be reinforced in PIN1At by the presence of a fourth basic residue, the lysine 22 that substitutes a strictly conserved proline residue in the other PIN-type PPIases. Unlike the experimental evidence for the human PIN1 PPIase activity, obtained on the basis of an indirect chymotrypsin-coupled assay, we used a direct functional assay based on the observation of interconverted molecules during the mixing time of a 2D EXSY NMR experiment. The well resolved signals for the cis and trans isomers of the peptide substrate showed that the uncatalyzed rate constant is too slow to be detectable. In the presence of 10 μM PIN1At, we measured an interconversion rate of 1 s⁻¹. This value is comparable with the 1.3 s⁻¹ rate measured in an analogous fashion for the cis/trans isomerization enhancement of the proline peptide bond of calcitonin by cyclophilin (24) and illustrates a similar catalytic efficiency of cyclophilins and parvulins. At this moment, we only dispose of data regarding the in vitro activity on a small peptide, but the in vivo protein substrates of the human PIN1 could well be conserved in A. thaliana. PIN1 and the mitotic protein monoclonal antibody MPM2 was shown to bind similar epitopes (5). The same antibody also recognizes plant antigens, suggesting the conservation in plants of an epitope that could be a PIN1At substrate (27). Further experiments are now needed to prove whether PIN1At is involved in the plant cell cycle and to find out how the absence of a WW domain influences the PIN1At activity and function. The question can even be raised whether there is another phosphorylation-dependent parvulin with a WW domain in A. thaliana, similar to the isomers known for cyclophilins and FK506-binding proteins or whether PIN1At is a new type of phosphorylation-dependent parvulin, specific or not, to plant organisms.

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The *Arabidopsis thaliana* PIN1At Gene Encodes a Single-domain Phosphorylation-dependent Peptidyl Prolyl cis/trans Isomerase

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