The recent crystal structure of Pin1 protein bound to a doubly phosphorylated peptide from the C-terminal domain of RNA polymerase II revealed that binding interactions between Pin1 and its substrate take place through its Trp-Trp (WW) domain at the level of the loop Ser\textsuperscript{11}-Arg\textsuperscript{12} and the aromatic pair Tyr\textsuperscript{18}-Trp\textsuperscript{29}, and showed a trans conformation for both pSer-Pro peptide bonds. However, the orientation of the ligand in the aromatic recognition groove still could be sequence-specific, as previously observed in SH3 domains complexed by peptide ligands or for different class of WW domains (Zarrinpar, A., and Lim, W. A. (2000) Nat. Struct. Biol. 7, 611–613). Because the bound peptide conformation could also differ as observed for peptide ligands bound to the 14-3-3 domain, ligand orientation and conformation for two other biologically relevant monophosphate substrates, one derived from the Cdc25 phosphatase of Xenopus laevis (EQPLLpTPVTDL) and another from the human tau protein (KUSVbRpTPPKSPS) in complex with the WW domain are here studied by solution NMR methods. First, the proton resonance perturbations on the WW domain upon complexation with both peptide ligands were determined to be essentially located in the positively charged β-hairpin Ser\textsuperscript{11}-Gly\textsuperscript{15} and around the aromatic Trp\textsuperscript{29}. Dissociation equilibrium constants of 117 and 230 μM for Cdc25 and tau peptides, respectively, were found. Several intermolecular nuclear Overhauser effects between WW domain and substrates were obtained from a ligand-saturated solution and were used to determine the structures of the complexes in solution. We found a similar N to C orientation as the one observed in the crystal complex structure of Pin1 and a trans conformation for the pThr-Pro peptidic bond in both peptide ligands, thereby indicating a unique binding scheme for the Pin1 WW domain to its multiple substrates.

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The \*H resonances of Pin1 WW domains 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 codes 1I8C for the WW domain, 1I8G for the complex Cdc25 peptide/domain WW, and 1I8H for the complex tau peptide/domain WW.

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\*\† The abbreviations used are: WW, Trp-Trp domain; CTD, C-terminal domain of RNA polymerase II; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; pSer/pThr or pS/pT, phosphoserine/phosphothreonine; r.m.s., root mean square; TOCSY, total correlation spectroscopy; SH, Src homology.
Phosphopeptide-WW Domain Binding Interactions

Structural statistics of the 20 favorable conformers

|             | WW          | WW-cdc25    | WW-τ        |
|-------------|-------------|-------------|-------------|
| Residues in most favorable regions (%) | 67.2 ± 6.7 | 61.7 ± 4.6 | 64.7 ± 4.8 |
| Residues in additional allowed regions (%) | 23.6 ± 5.9 | 18.7 ± 6.8 | 24.5 ± 5.0 |

TABLE I

Restrains for calculations
Total restraints used (i = j)

|              | WW          | WW-cdc25    | WW-τ        |
|--------------|-------------|-------------|-------------|
| 643          | 568         | 562         |
| Intrareside (i = j) | 221         | 214         | 215         |
| Sequential (i = j + 1) | 164         | 161         | 154         |
| Medium range (1 < |j| - j < 5) | 90          | 72          | 73          |
| Long range (i = j > 4) | 165         | 121         | 120         |
| Hydrogen bond restraints | 22          | 22          | 22          |
| Intermolecular restraints | 0          | 22          | 14          |
| r.m.s. deviations from experimental NOE restraints (Å) | 0.0100 ± 0.0004 | 0.0466 ± 0.0001 | 0.0562 ± 0.0025 |
| r.m.s. deviations from idealized geometry | 0.20 ± 0.03 | 1.67 ± 0.23 | 1.63 ± 0.25 |
| Bonds (Å) | 0.0051 ± 0.0007 | 0.0065 ± 0.0001 | 0.0045 ± 0.0004 |
| Angles (°) | 0.84 ± 0.09 | 0.71 ± 0.02 | 0.68 ± 0.03 |
| Improper (°) | 0.74 ± 0.03 | 0.60 ± 0.02 | 0.61 ± 0.04 |
| Ramachandran plot (PROCHECK-NMR) | 0.02 0.61 | 0.02 0.68 | 0.03 0.68 |

‡ No distance restraint in any of the structures included in the ensembles was violated by more than 0.5 Å.

† Idealized geometries based on X-PLOR parameters (parallhdg.pro).

‡‡ Only the coordinates of residues Pro⁴ to Pro³² of the WW domain were considered to exclude the disordered N and C termini.

bind peptidic ligands in both orientations (15). It therefore remains an open question whether the group IV WW domain of Pin1 is capable of binding different ligands in different orientations or whether a unique orientation is preferred.

Furthermore it is of major interest to consider the conformation of the recognized motif and, more specifically, the isomerization state of the peptide bond in the pSer/pThr-Pro motif. In the crystal structure of Pin1 complex (13), the peptide ligand binds with both pSer-Pro peptide bonds in trans conformation. The cis conformation of the peptide would require major rearrangements of the binding site and is therefore not very probable. However, for a related adapter module, the 14-3-3 domain, a recent crystallography study showed that peptide substrates can be bound with both isomerization states (cis and trans) (16).

Here we have investigated by solution NMR the binding interactions between a synthetic Pin1 WW domain and two biologically relevant monophosphorylated substrates, a Cdc25 peptide model (EQPLpTPVTL) and a peptide of the τ protein (KVSVRpTPPKSPS). Based on the structures of both complexes in solution, we found a similar binding mode as the one observed in the crystal structure of Pin1 bound to a peptide isolated from the CTD of RNA polymerase II (13). This observation gives experimental evidence to a unique binding scheme for Pin1 WW domain to its multiple substrates. Finally, we discuss the implication of this aspect in regard of recent data concerning Pin1 molecular function (17).

EXPERIMENTAL PROCEDURES

Peptide Synthesis—The Pin1 WW domain was obtained by peptide synthesis using the t-butoxycarbonyl-benzyl strategy and the (2,1H-benzotriazolyl-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate in situ activation protocol on an Applied Biosystems 430A peptide synthesizer (Foster City, CA). Side-chain protections were Arg(Tos), Trp(For), Asn(Trt), Gln(Trt), Ser(Bzl), His(Tos), Tyr(Br-Z), Lys(2-CIZ). The formyl group of tryptophan was removed with a solution of 20% piperidine in dimethyl formamide prior to hydrofluoric acid cleavage. After lyophilization, the crude peptide was purified by reverse phase on an X-terra RP18 (Waters, Milford, MA) and mass spectrometry on a Quattro II electrospray mass spectrometer (Micromass, Manchester, UK), were pooled and lyophilized. The same conditions and procedures were used for WWR12A and WWS11A mutants.

The phosphopeptide sequences, derived from Cdc25 (AcEQPLpTPVTL) and τ (AcKVSVRpTPPKSPS) protein sequences, were previously identified by peptide-scanning experiments as major binding sites for the Pin1 WW domain (7, 11) and were also supplied by peptide synthesis. Peptide chemical synthesis was identical as described (18).

NMR Spectroscopy—Experiments were performed on a Bruker DMX 600-MHz spectrometer and processed with the Bruker software. Spectra were recorded on a 1 mm sample of the WW domain in a buffer of 50 mM deuterated Tris-HCl, pH 6.4, 100 mM NaCl and at a temperature of 12 °C. Two-dimensional TOCSY (19) and NOESY (20) spectra were acquired with mixing times of 62 and 300 ms, respectively. Spectral widths were 8400 Hz, with 1024 complex points for t2, 256 complex points for t1, 32 transients per increment for TOCSY experiments, and 64 transients for NOEY experiments. The water signal was suppressed using the Watergate (21) or jump-return (22) pulse sequences. The data sets were processed with squared sine functions, and zero filling was applied to obtain spectra with a digital resolution of 16.4 Hz/point in the F1 dimension and 8.2 Hz/point in the F2 dimension. The classical homonuclear NMR procedure for resonance assignment in polypeptides was used (23). The stereospecific assignment was assisted by chemical shift calculations (24) computed from the x-ray structure of Pin1 WW domain (Rutgers University Protein Data Bank code 1PIN (25)). Proton chemical shifts were measured in parts per million (ppm) and referenced relative to the methyl proton resonances (put at 0.0 ppm) of the internal standard trimethyl silyl propionate.

NMR Titration—Increasing amounts of phosphopeptide ligands, with Cdc25 or τ sequences, were added to a 1 mM sample of WW domain to give molar ratios ranging from 1:0.0 to 1:4.5 for the WW domain-Cdc25 peptide sample or from 1:0.0 to 1:11.0 for the WW domain-τ peptide sample. The pH of each sample was systematically controlled and, if necessary, adjusted to a value of 6.4 by addition of an aliquot of dilute NaOD or DCl solutions. For each titration point, a one-dimensional NMR spectrum was recorded. In addition, two-dimensional NMR TOCSY and NOEY experiments were acquired at low ligand concentration (typically at a ratio of 1:9.5), at equimolar concentration and finally at substrate saturation. For the latter ratio, NOEY spectra with a high number of scans per increment (i.e. 416 and 320, respectively, for the WW domain-Cdc25 and WW domain-τ samples) were acquired, to detect possible weak intermolecular NOEs. The binding constant was calculated by fitting to the following equation,

$$\Delta \delta = 0.5 \Delta \delta_{\text{max}} (1 + X + K_d / [WW]) $$

$$- [(1 + X + K_d [WW])^2 - 4X]^{0.5}$$

(1) where $\Delta \delta$ is the observed chemical shift, $\Delta \delta_{\text{max}}$ is the maximum shift at ligand saturation, [WW] is the protein concentration, $K_d$ is the dissoci-
RATION constant, and \( x \) is the molar ratio of peptide substrate to protein concentration \([\text{WW}].\)

**Solution Structure Calculations**—Three-dimensional models of WW domain and of complexes between phosphothreonine peptides and WW domain were determined with interproton restraints derived from NOE spectra. NOEs within the protein and between the protein and ligand were grouped into four distance ranges: 1.8–3.0, 1.8–4.0, 2.0–5.0, and 2.5–6.0 Å, corresponding to strong, medium, weak, and very weak NOEs, respectively. To account for the higher apparent intensity of methyl resonances, 0.5 Å was added to the upper distance limits for NOEs involving methyl protons. Protein backbone hydrogen-bonding restraints (two per hydrogen bond; \( r_{\text{HN-O}} = 1.5–2.5 \text{ Å} \); \( r_{\text{HN-N}} = 2.5–3.5 \text{ Å} \)) with the experimental intermolecular NOEs as distance constraints. The minimization procedure consisted of 50 steps of steepest descent followed by steps of Quasi-Newton-Raphson optimization (29) until the maximum energy derivative was 0.01 Å and 2°, respectively, were further submitted to a restrained energy refinement. CVFF was used as a force field in the Discover modules (MSI software) (28), with the experimental intermolecular NOEs used as distance constraints. The stereochemical quality of the structures was evaluated by the PROCHECK-NMR program (30).

**RESULTS**

**NMR Assignment and Structure of the Isolated WW Domain**—Sequence-specific \(^1\text{H}\) NMR assignments of the WW domain, based on homonuclear TOCSY and NOESY spectra recorded in \( \text{H}_2\text{O} \) (23), were straightforward due to the large \(^1\text{H}\) NMR chemical shifts dispersion observed, ranging from 10.57 ppm for the Hn1 of Thr to the most upfield resonance for the Hb1 of Asn at \(-0.64 \text{ ppm} \). Chemical shift calculations starting from the WW domain x-ray structure confirmed the atypical resonance frequencies of several protons and indicated the ring current shifts associated with the high number of aromatic residues in the WW domain as the primary source. In particular, the chemical shifts of protons Asn\(^2\) H\(\beta\) (\(-0.64 \text{ ppm} \)), Asn\(^1\) H\(\delta\) (4.23 ppm), Pro\(^2\) H\(\alpha\) (0.03 and 0.62 ppm), and Pro\(^3\) H\(\gamma\) (0.79 and 0.87 ppm) showed large upfield shifts due to the ring current effect of the Thr indole ring. In the same way the proximity of the aromatic rings of Tyr\(^{29}\) and Tyr\(^{39}\) contributed to the upfield shifts of protons Arg\(^2\) H\(\beta\) (0.10 ppm) and Arg\(^1\) H\(\alpha\) (2.68 ppm), respectively.

On the basis of 643 experimental NOE constraints, we derived the solution structure of the WW domain. The structural and energetic statistics on the 20 favorable WW conformers are shown in Table I. The overall fold of the synthetic WW peptide, an antiparallel \( \beta \)-sheet composed of three \( \beta \)-strands, was very similar to the x-ray crystallography model (Protein Data Bank code 1PIN (25)), with an r.m.s. backbone deviation of 1.38 Å.

**NMR Titration with Ligands**—The binding interaction between phosphothreonine peptides and the WW domain was examined by means of one-dimensional \(^1\text{H}\) NMR spectra (Fig. 1A). Addition of increasing amounts of peptide ligands caused chemical shift changes for several protons in the WW domain. During the NMR titration some proton resonances, such as the Hn of Arg\(^2\) and the Hn1 of Thr\(^{29}\) rapidly became broader, until they disappeared from the spectra to reappear at large excess of ligand. This broadening can be ascribed to slow exchange, with the difference in chemical shift for the proton concerned in its free and bound state being of the same order as the exchange rate (31). Other resonances, such as the amide proton of Ser\(^1\), moved gradually, whereas many others exhibited practically no chemical shift perturbations. Resonance frequencies for protons of the WW domain were similarly affected upon binding of the Cdc25 and p peptide ligands, indicating an equivalent binding mode for both phosphothreonine peptides. The isolated Ser\(^1\) amide proton resonance, in rapid exchange on the NMR time scale and hence apparent throughout the titration, was used to estimate a binding constant of 117 and 230 \( \mu \text{M} \) for the Cdc25 and p peptide ligands, respectively (Fig. 1B). Finally, the non-phosphorylated variants of peptide ligands did not induce modifications of the WW domain resonances (data not shown), confirming the phospho-dependence of the interaction.

**NMR Binding Maps**—To obtain a precise description of the complex, two-dimensional NMR spectra of the ligand-saturated WW protein were recorded and completely reassigned. Comparison with the assignments of the free WW domain allowed a precise mapping of the binding surface (Fig. 2). The chemical shift perturbations were essentially grouped in two regions of
the WW domain: the loop region between the strands β1 and β2 (Ser¹¹-Gly¹⁵) and the C terminus of the strand β3 (Glu²⁹-Glu³⁰). The largest chemical shift changes were observed for the Hn1 of Trp²⁹ and the backbone amide proton of Arg¹², indicating thereby their presence in the immediate vicinity of the binding site. Noteworthy, an exceptional value of 11.4 ppm was found in the bound form for the resonance of Arg¹² backbone amide proton. When displayed on the WW domain surface (Fig. 2), those two most perturbed regions delineated in an unambiguous fashion the phospho-ligand binding site.

The fact that the WW domain folds into a small compact structure and that only few residues were strongly affected by addition of phosphopeptides suggests that chemical shift changes were caused by direct interaction between the WW domain and peptide ligands and not by some ligand-induced conformational changes. The β-sheet twist modification upon phosphopeptide complexation, reported from x-ray crystal data (13), appears thus as a minor conformational change during the binding leading to no substantial changes in the pattern of intramolecular NOEs.

Because the key role of Trp²⁹ in the binding feature was previously established for other WW domains (3, 12), we focused on the influence of the loop Ser¹¹-Gly¹⁵. Two WW domain mutants, Ser¹¹→Ala and Arg¹²→Ala, were synthesized. After confirmation of their structural integrity by circular dichroism and NMR spectra (data not shown), we performed an NMR titration with the Cdc25 phospho-peptide. For both mutants, we observed chemical shift changes upon addition of phosphorylated peptide (Fig. 3), confirming their capacity to bind the Cdc25 peptide. However, chemical shift perturbations were less important, and a 3-fold excess of peptide was not sufficient to drive the Arg¹² amide proton resonance of the Ser¹¹→Ala WW mutant into the fast exchange regime with concomitant line narrowing (Fig. 3). Therefore, mutations in the loop appeared to weaken the interaction without destroying it completely. The binding affinity between phospho-substrates and Pin1 WW domain seems thus generated by a sum of at least two energetically favorable interactions, the charge-charge interaction between the phosphate group and the positively charged WW loop and the proline- aromatic interaction, with none of the individual interactions appearing as absolutely essential (13).

Conformation of the Bound Peptide—The conformation of the recognized motif on the peptide ligands and, more specifically, the isomerization state of the peptide bond separating the phospho-residue and proline, is of great interest. We set out to distinguish both isofoms first in the free peptides and then in the complexes. For the τ peptide, the cis form of the Pro⁶ following the pThr⁷ residue could not be detected, in agreement with a recent study that gave an upper limit of 3% for the cis form of the same Pro residue in a somewhat longer peptide that was phosphorylated on Thr⁷ and Ser¹¹ (32). For the Cdc25 phosphothreonine peptide (right side) at a protein:ligand molar ratio of 1:3. Chemical shift perturbations for the Hn1 of Trp²⁹ and the backbone amide proton of residue 11 are larger in the wild type WW domain than in both mutants.

Intermolecular NOE Data and Complex Structures—On the basis of NOESY analysis on a ligand-saturated WW sample, 14

### Figure 2
Plot of backbone amide chemical shift changes observed for each residue of WW domain in the presence of a large excess of Cdc25 or τ peptide ligand. The three β-strands constituting the WW domain fold are shown by arrows. The very large shift (2.10 ppm) of Hn-Arg¹² resonance is indicated. On the left side, a ribbon drawing of the WW domain, with residues displaying the largest chemical shifts rendered in detail, is shown. Note that due to some overlapped resonances we were not able to unambiguously assign chemical shifts of Ser¹³ and Ser¹⁴ in the bound form with τ ligand.

### Figure 3
1H NMR spectrum of amide regions for wild type WW mutants (bottom), Arg¹²→Ala mutant (middle), and Ser¹¹→Ala mutant (top), either in the solution free state (left side) or bound to Cdc25 phosphothreonine peptide (right side) at a protein:ligand molar ratio of 1:3. Chemical shift perturbations for the Hn1 of Trp²⁹ and the backbone amide proton of residue 11 are larger in the wild type WW domain than in both mutants.
and 22 unambiguous protein-ligand NOEs were identified for the Cdc25 and for the τ peptide, respectively. Contacts with pThr2, Pro6, and Pro9 of the τ peptide represent intermolecular NOEs that helped to determine the orientation of the peptide ligand in the recognition site of the WW domain. Other intermolecular NOEs were detected between protons of the WW Arg6, Arg12, Phe20, and Trp29 residues and protons of pThr5, Pro6, and Val7 of the Cdc25 peptide.

These NOEs sets were translated into intermolecular proton-proton distances and formed the basis for construction of the structure of the complexes. Table I gives the structural statistics of the complex models. The WW domain conformation was perturbed by the interaction with the ligand, but the main features of the complex remain intact. The substrate phosphate moiety is bound to the side chain of arginine Arg12 of the WW domain.

The reversible protein phosphorylation on Ser/Thr-Pro motifs acts as an important molecular switch in the cell cycle and is operated by a number of Pro-directed protein kinases (such as Cdc2, Cdk2, Wee1, and Myt1; for a review, see Ref. 33) and phosphatases, such as Cdc25 and PP2A (34, 35). Although atomic level structural data of all substrates in their phosphorylated and non-phosphorylated state are still lacking, one example of the structural influence of phosphorylation is given by Cdk2 itself, where addition of a phosphate group on the Thr160 side chain completes the reorganization of the substrate binding site that was started by cyclin binding (36). A second mechanism of imposing structural changes is brought about by the isomerization of the prolyl peptide bond, and a prolyl cis/trans isomerase, Pin1, has been shown to be essential to achieve a cell cycle (37, 38). A remarkable fact of both Cdk2 and Pin1 is that they associate with small binding modules that are believed to help targeting the catalytic domain more efficiently to their substrates. Cdk2 recruits the CKS, that indeed binds through its anion binding site to the pThr-Pro motif of a Cdc25 peptide (39), whereas human Pin1 contains in its sequence itself already such a binding protein, the WW domain (7, 37). Both functional (40) and structural (39) data indicated a molecular competition of both of binding modules for the same Cdc25 substrate, and this competition indeed might be part of the tight regulation of the cell cycle.

In a related cellular process, both Cdk2 and Pin1 interact with the τ protein. Cdk2 phosphorylates τ proteins, in an Alzheimer-like way, leading to microtubule depolymerization (41). Conversely, Pin1 can restore the tubulin polymerization of the prolyl peptide bond, and a prolyl cis/trans isomerase, Pin1, has been shown to be essential to achieve a cell cycle (37, 38). A remarkable fact of both Cdk2 and Pin1 is that they associate with small binding modules that are believed to help targeting the catalytic domain more efficiently to their substrates. Cdk2 recruits the CKS, that indeed binds through its anion binding site to the pThr-Pro motif of a Cdc25 peptide (39), whereas human Pin1 contains in its sequence itself already such a binding protein, the WW domain (7, 37). Both functional (40) and structural (39) data indicated a molecular competition of both of binding modules for the same Cdc25 substrate, and this competition indeed might be part of the tight regulation of the cell cycle.

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concentration in the cell, our calculated $K_d$ appears more relevant, and confirms that the interaction of Pin1 with Cdc25 and $\tau$ are related molecular processes.

Despite the reported selectivity of Pin1, we found that both Cdc25 and $\tau$ phosphopeptides bind to Pin1 WW domains in a similar manner, close to the described binding pattern in the CTD peptide-Pin1 x-ray model (13). However, the crystal complex of the CTD peptide-Pin1 protein shows a larger contact surface between protein and ligand than what was observed in our NMR models. The anchoring zone of the Cdc25 and $\tau$ peptide ligands to the WW domain was only composed of the phospho-residue and residues 11 and 12 (Figs. 5 and 6). We did not observe any significant interaction with the Tyr18 of the WW domain, neither in the chemical shift perturbation assay, nor by the observation of intermolecular NOEs.

The three-dimensional NMR complex model shows that the phosphopeptide ligands are principally fixed by a charge-charge interaction and a proline-aromatic stacking. The participation of the backbone amide group in complexation of the phosphate moiety could explain partly why the mutant Arg$^{12} \rightarrow$ Ala persists to fix ligand. The same argument could help to interpret the phosphopeptide binding properties of other Pin1-related WW domains, such as Ned44 or Rsp5 proteins, that lack a basic residue in the $\beta_1$-$\beta_2$ loop. In addition, it must be stressed that the intermolecular stacking structure between Trp$^{29}$ and the proline at position +1 of pThr ligand is analogous to the intramolecular stacking between conserved residues Trp$^6$ and Pro$^{32}$ of domain WW (Fig. 6). Because several mutagenesis studies (10, 43–45) have established the importance of the Trp$^6$-Pro$^{32}$ interaction for the folding and the stability of WW domains, we think that a similar strong intermolecular Trp-Pro interaction is used to promote protein-protein interactions between the WW domain and its substrates (46).

The molecular mechanisms of the Pin1 function very recently became clearer through an elegant study by Zhou et al. (17), who showed that Pin1 enhances in vitro the dephosphorylation of Cdc25C by the major trans-Pro-directed phosphatase PP2A. Moreover, they showed that overexpression of the catalytic domain alone can be sufficient for cell survival, whereas at lower levels of expression this is not the case (7). At the molecular level, Pin1 would catalyze the conversion of the cis bond in the pThr-Pro motif of Cdc25C, turning the peptide into a suitable substrate of PP2A. Although direct evidence of such a cis peptide bond in the complete Cdc25 or $\tau$ lacks, their biochemical evidence that Pin1 up-regulates the dephosphorylation of both Cdc25 and $\tau$ convincingly sustains this hypothesis. However, the results of our NMR study unambiguously define the pThr-trans-Pro motif in both Cdc25 and $\tau$ peptide ligands as the binding surface recognized by the Pin1 WW domain, in agreement with the earlier crystal structure of full Pin1 with a doubly phosphorylated CTD peptide (13). Moreover, because our NMR results confirm the absence of interaction between

![Ribbon drawing of the NMR reference structure of the complex between Pin1 WW domain (in light blue) and $\tau$ phosphopeptide ligand (in red), in comparison with the orientation of the CTD peptide (in violet) from the crystallographic model of the complex (13). The image was obtained by backbone superimposition of WW domains from our NMR complex and from the CTD peptide/Pin1 complex (13). Only the WW domain from this study and both phosphopeptide ligands are represented. Side chains implicated into the binding interface are labeled (in white for the WW domain residues and yellow for the ligand residues) and depicted in detail, as well as the amino acid pair Trp$^{6}$-Pro$^{32}$ of the WW domain. N atoms are blue and P atoms are violet. C atoms are green in the WW domain and orange in the tau ligand.](image)
the Pro residue in the cis conformation and the WW domain, it is unlikely that the latter would target the catalytic domain by targeting it to its pThr/Ser-cis-Pro substrate, be it on Cdc25, τ or other protein targets.

The available data can be combined into several scenarios. The WW domain could specifically target Pin1 to the pThr²³¹ of τ or pThr⁴⁷ and pThr⁶⁸ of Cdc25, followed by isomerization of neighboring pThr-Pro bonds through the catalytic domain (47). In another scenario, the catalytic domain could induce the conformational transition from cis to trans of the pThr-Pro motif prior to any WW binding. Before a return to the cis/trans equilibrium situation, the WW domain could then immediately bind to the pThr-trans conformation, thereby stabilizing this conformation and simultaneously increasing the local concentration of Pin1. Both scenarios solve the dilemma that the WW domain has an at least 10-fold higher affinity as does the catalytic domain for the same substrates (7, 13) and, consequently, would bind first to the epitope before catalysis could happen. Further studies will be necessary to investigate this possibility of prolyl cis/trans isomerization anterior to WW binding.

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REFERENCES
1. Bork, P., and Sudol, M. (1994) Trends Biochem. Sci. 19, 531–533
2. Staub, O., and Rotin, D. (1996) Structure 4, 495–499
3. Macias, M. J., Hyvönen, M., Baraldi, E., Schultz, J., Sudol, M., Saraste, M., and Oshchikin, H. (1996) Nature 382, 646–649
4. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1315–1327
5. Kay, B. K., Williamson, M. P., and Sudol, M. (2000) J. Biol. Chem. 275, 17070–17077
6. Bedford, M., Sarbassova, D., Xu, J., Leder, P., and Yaffe, M. B. (2000) J. Biol. Chem. 275, 10359–10369
7. Lu, P.-J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) Cell 97, 8686–8687
8. Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Kelly, J. W., Meier, B. H., Bachmann, P., and Ernst, R. R. (1999) J. Am. Chem. Soc. 121, 7310–7311
9. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) J. Mol. Biol. 283–292
10. Crenshaw, D. G., Yang, J., Means, A. R., and Kornbluth, S. (1998) EMBO J. 17, 1315–1327
11. Lu, P.-J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) Nature 399, 784–788
12. Koepf, E. K., Petrassi, H. M., Ratnaswamy, G., Huff, M. E., Sudol, M., and Kelly, J. W. (1999) Biochemistry 38, 14338–14351
13. Verdecia, M. A., Bowman, M. E., Lu, K. P., Hunter, T., and Noel, J. P. (2000) Nat. Struct. Biol. 7, 639–643
14. Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M., and Eck, M. J. (2000) Nat. Struct. Biol. 7, 634–638