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Modulating the Affinities of Phosphopeptides for the Human Pin1 WW Domain Using 4-Substituted Proline Derivatives

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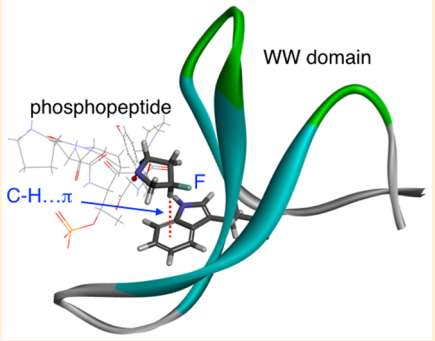
Supporting Information

ABSTRACT: Human Pin1 is involved in cancer developments and has been a pharmaceutical target. Thus, finding a high-affinity inhibitor of Pin1 has become an attractive topic. The WW domain of human Pin1 can recognize the phosphoserine/phosphothreonine-proline (pS/pT-P) motifs, while its PPIase domain catalyzes the *cis*/*trans* isomerization of prolyl bonds to regulate the cell cycle. Here we incorporated a series of 4-substituted proline derivatives into the phosphopeptides and investigated their affinities for the WW domain of Pin1 to develop better inhibitors of Pin1. On the basis of the ligand Myt1-T412 [PPA(pT)PP], we synthesized several phosphopeptides in which the proline residue in the pT-P motif was replaced with various 4-substituted proline derivatives. Isothermal titration calorimetry and fluorescence anisotropy analyses show that the replacement of proline with (2*S*,4*R*)-4-fluoroproline increases the binding affinity of the peptide. Circular dichroism measurements suggest that a more PPII-like structure of phosphopeptides makes them bind to the WW domain more tightly. Chemical shift perturbation experiments also indicate that (2*S*,4*R*)-4-fluoroproline interacts with Trp34 of the WW domain in the binding site. Results of molecular modeling further suggested that a strong C−H···π interaction induced by (2*S*,4*R*)-4-fluoroproline is important in enhancing the affinity of the peptide for the WW domain. The results of this study provide new valuable information for designing and developing effective inhibitors of human Pin1.



Suppressing the function of specific proteins that activate Ras alleles to promote oncogenesis is the main therapy for cancers. One of these proteins is peptidyl-prolyl *cis*/*trans* isomerase NIMA-interacting 1 (Pin1), which could activate Ras alleles to cause cancers.[1−3] Pin1 mainly isomerizes *cis*/*trans* conformers of phospho-Ser/Thr-Pro (pS/pT-P) bonds. It contains two domains: (1) the WW domain that binds to substrates containing pS/pT-P motifs and (2) the PPIase domain that catalyzes the isomerization of the prolyl bonds.[4] The WW domain of Pin1 is the key module that recognizes phosphoproteins,[4−6] because the other human parvulin, hPar14, does not contain the WW domain and cannot catalyze phosphoproteins.[7] Pin1 also contributes to the major PPIase activity against phosphorylated substrates in mammalian cells,[8] and its function is associated with tumor maintenance.[9] Thus, use of effective inhibitors of Pin1 could be a potential approach for cancer treatments.

Various Pin1 inhibitors have been reported in the literature, such as Juglone,[10] dipentamethylene thiauram monosulfide,[11] D-peptide-based ligands,[12] cyclic peptides,[13−16] conformation-locked alkene peptidomimetics,[17] reduced amide inhibitors mimicking the transition state,[18] and EGCG, a major flavonoid in green tea.[19] In addition, the conformation-locked inhibitors were studied by nuclear magnetic resonance (NMR) dynamics,[20,21] and the binding interactions of Pin1 with the reduced amide inhibitor, the D-peptides, and the locked alkenes were determined by X-ray crystallography.[18,22,23] The mode of inhibition of the locked alkenes was also determined to be competitive by extensive kinetic inhibition assays.[17] These results provided valuable information for the development of an appropriate inhibitor for Pin1. Furthermore, Pin1 was shown to be inhibited by phosphorylation at Ser16, which prevents the WW domain from binding to phosphopeptides,[24] implying that disrupting the interaction between the WW domain and phosphopeptides could effectively inhibit Pin1 activity. On the basis of the results, Déprez and co-workers synthesized a series of ligands by introducing hydrophobic moieties into the pT-P dipeptide, tracked their binding affinity for the WW domain using $^{1}H-^{15}N$ HSQC NMR spectra,[25] and found that a few dipeptides showed a high affinity for the WW domain. However, the details about the relationship between peptide structure and binding affinity were not provided in this work. Thus, it inspired us to pursue more insights into the interactions between phosphopeptides and the WW domain. Recently, Zondlo and co-workers also showed that phosphorylation of a peptide increased the level of its polyproline II

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(PPII) conformation and suggested that PPII structure could assist the binding of a peptide to the Pin1 WW domain.[26] According to these results, we decided to explore how the conformation of a phosphopeptide affects its binding affinity for the WW domain as the basis to design an effective inhibitor of Pin1.

Of the reported peptide substrates of the Pin1 WW domain, we chose a proline-rich peptide, Myt1-T412 [PPA(pT)PP],[27] as the parent peptide. Previous studies have shown that 4-substituted proline derivatives could modulate PPII forming propensities.[28−31] Moreover, our recent study showed that stabilization of the WW domain by incorporating (2*S*,4*S*)-4-fluoroproline in place of Pro37 enhanced its affinity for the phosphopeptide even though the substitution site was not in the binding site, suggesting that these 4-substituted proline derivatives could mediate the binding between the WW domain and phosphopeptides.[32] In this study, we used six 4-substituted proline derivatives, namely, (2*S*,4*R*)-4-hydroxyproline (Hyp), (2*S*,4*R*)-4-fluoroproline (Flp), (2*S*,4*R*)-4-methoxyproline (Mop), (2*S*,4*S*)-4-hydroxyproline (hyp), (2*S*,4*S*)-4-fluoroproline (flp), and (2*S*,4*S*)-4-methoxyproline (mop), to replace Pro in the pT-P motif of Myt1-T412, and examine their affinities for the WW domain.

ITC analysis and a fluorescence anisotropy assay indicate that among the derivatives used, only Flp enhances the affinity of the peptide for the WW domain. Chemical shift perturbation measurements show that the WW domain interacts with the Flp-containing peptide through the same interface as that used while interacting with a Pro-containing peptide. Circular dichroism (CD) measurements also indicate that the Flp-containing peptide forms the most PPII-like structure, suggesting that the affinity of the peptide is correlated with its PPII forming propensity. We further used molecular modeling to learn that the positive impact of Flp on the binding affinity could be attributed to the strong C−H···π interaction between Flp and Trp34 of the WW domain, which is induced by the electron-withdrawing fluorine atom on the pyrrolidine ring. The findings of this study provide new insights into the interactions between phosphopeptides and the WW domain, which could be used in the future development of Pin1 inhibitors.

MATERIALS AND METHODS

General. Fmoc-(2*S*,4*R*)-4-fluoroproline was purchased from Bachem Bioscience, and Fmoc-protected phosphothreonine was purchased from Merck. The reagents and Fmoc-protected amino acids purchased were directly used without further purification. Fmoc-(2*S*,4*S*)-4-fluoroproline, Fmoc-(2*S*,4*S*)-4-hydroxyproline, Fmoc-(2*S*,4*R*)-4-methoxyproline, and Fmoc-(2*S*,4*S*)-4-methoxyproline were synthesized as described previously.[28,29] The syntheses of Fmoc-protected proline derivatives were monitored by a Varian 400-MR NMR spectrometer. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were recorded using an Autoflex III Smartbeam LRF200-CID spectrometer (Bruker Daltonics).

Purification of the Human Pin1 (hPin1) WW Domain. The hPin1 WW domain and the ^{15}N-labeled hPin1 WW domain were expressed in *Escherichia coli* as fusion proteins with glutathione *S*-transferase (GST) at 37 °C and purified by affinity chromatography with glutathione-sepharose. The GST tag was removed by thrombin digestion at 25 °C for 12 h. The Pin1 WW domain was separated from GST and thrombin by reverse-phase high-performance liquid chromatography (HPLC) using a Vydac semipreparative C18 column. H_2O/acetonitrile gradients with 0.1% (w/v) TFA as the counterion were used for the eluting solvent system. Molecular masses of the proteins were verified by MALDI-TOF mass spectrometry.

Peptide Synthesis and Purification. All peptides were prepared on a 0.05 or 0.1 mmol scale by standard solid-phase methods and Fmoc chemistry protocols using Fmoc-protected amino acids and HBTU-mediated coupling reactions. Fluorescein-labeled peptides were synthesized according to a method adapted from a previous report.[33] The syntheses were conducted on a Discover SPS microwave peptide synthesizer (CEM Corp.). Use of a Rink amide resin generated an amidated C-terminus upon cleavage from the resin with a 92.5% trifluoroacetic acid (TFA)/2.5% ethanedithiol/2.5% triisopropylsilane/2.5% H_2O mixture (v/v). All the peptides were purified by reverse-phase HPLC with a Vydac semi-preparative C18 column. H_2O/acetonitrile gradients with 0.1% (w/v) TFA as the counterion were used to elute the peptides. The purity of the purified peptides was >90% according to HPLC analysis. The HPLC chromatograms of these purified peptides are shown in the Supporting Information (Figures S1−S3). Identities of all the peptides were confirmed by MALDI-TOF mass spectrometry. The measured mass data are shown in the Supporting Information (Table S1).

Circular Dichroism (CD) Spectroscopy. CD experiments were conducted on an Aviv model 410 CD spectrometer with a 1 mm path length quartz cuvette. Far-UV CD spectra were recorded with a peptide concentration of 200 μM in pH 7.1 and 20 mM sodium phosphate buffer at 25 °C.

Fluorescence Anisotropy. Fluorescence anisotropy measurements were acquired on a Chronos-FD spectrofluorimeter (ISS) with excitation and emission wavelengths of 492 and 519 nm, respectively. The peptides with 5(6)-carboxyfluorescein at the N-terminus were used for the measurements. Binding affinity measurements were taken in pH 7.1 and 20 mM sodium phosphate buffer at ambient temperature (~25 °C), with a final fluorescein-labeled peptide concentration of 2 nM. Anisotropy (r) values were calculated from the fluorescence intensity (I) and gain (G) in parallel and perpendicular modes using the following relationship:[34,35]

$$r = \frac{R_V - R_H}{R_V + 2R_H}$$

where

$$R_H = \frac{G_{\parallel} I_{\parallel}}{G_{\perp} I_{\perp}} \text{ and } R_V = \frac{G_{\parallel}}{G_{\perp}}$$

Triplicate measurements were conducted at each concentration, and the error bars in anisotropy are shown in the plots. Dissociation constants (K_d) were determined by fitting the binding curves of relative anisotropy versus protein concentration to a single-ligand binding model (as shown in the following equation) with GraphPad Prism 5 (GraphPad, La Jolla, CA).

$$r = \frac{r_{max} x^h}{K_d{}^h + x^h}$$

where r_{max} is the maximum anisotropy, x the concentration of protein, and h the Hill slope.

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NMR Spectroscopy. NMR spectra were recorded at 25 °C on a Varian 700 MHz spectrometer equipped with a cryo-probe (National Tsing Hua University Instrumentation Center). For the two-dimensional heteronuclear experiments (^{1}H−^{15}N HSQC), the concentration of the proteins was approximately 0.4 mM. Protein samples were prepared in 20 mM phosphate buffer (pH 7.1, 90% H_2O, 10% D_2O). The ^{15}N-labeled proteins were titrated with peptide ligands by a molar ratio of 1:0, 1:0.25, 1:0.5, 1:0.75, 1:1, and 1:2. The weighted average of the ^{15}N and ^{1}H chemical shift perturbations was calculated using the equation $\Delta\delta = [(\Delta\delta_H)^2 + 0.2(\Delta\delta_N)^2]^{1/2}$. The spectra were processed with Vnmr software and analyzed with SPARKY.[36] The backbone amide nitrogen and proton chemical shift assignments were achieved according to Biological Magnetic Resonance Data Bank (entry 17545).

Isothermal Titration Calorimetry (ITC). Binding affinities of the phosphopeptides for the WW domain were measured by a calorimetric assay with an ITC-200 instrument (MicroCal, Northampton, MA). The pH 7.1 and 20 mM sodium phosphate buffer was used for ITC measurements, and the titrations were conducted at 25 °C. A 2 mM ligand solution in the syringe was titrated into the ITC cell (volume of ∼0.3 mL) containing a solution of 0.2 mM protein. The titration heats were corrected by subtracting the heats of titrating the ligand into buffer. The binding constants were determined by performing calorimetric analyses with a one-site model of the Origin package (version 7) supplied with the instrument.

Molecular Modeling and Energy Minimization. Structure simulation and energy minimization were conducted using the Discovery Studio 4.0 program provided by the National Center for High-Performance Computing (NCHC, Taiwan). The initial structure of the WW domain−peptide complex was taken from the Protein Data Bank (PDB entry 1F8A). The original ligand in the structure was modified to our peptide sequence and proline derivatives used. The CHARMm force field was applied in the simulation, and the computation was performed in the presence of solvent (water molecules). We used a harmonic constraint of 5, 10, and 20 kcal/mol to minimize the ligand-bound structure. After the minimization with constraints, the conformation was further energy-minimized without constraints to give the final optimized structure.

RESULTS AND DISCUSSION

Design and Synthesis of Phosphopeptides and Preparation of the WW Domain. For human Pin1, its WW domain can recognize the pS/pT-P motif, and the PPIase domain catalyzes the isomerization of peptide bonds in the substrates to regulate the cell cycle.[37,38] A previous study showed that the proline-rich region of the peptide substrate adopted a polyproline II (PPII) helix and promoted the binding of the substrate to the WW domain.[27] An electron-withdrawing substituent (e.g., OH, F, and OMe) in the 4*R* or 4*S* position of proline induces a different preference of C$^\gamma$-ring pucker, *cis*−*trans* ratio of prolyl peptide bonds, and PPII conformation in which 4*R*-substituted proline derivatives (Hyp, Flp, and Mop) favor a C$^\gamma$-*exo* pucker and a *trans* prolyl peptide bond while their 4*S*-diastereomers (hyp, flp, and mop) prefer a C$^\gamma$-*endo* pucker and induce more *cis* prolyl peptide bonds than proline. Therefore, we used these derivatives to replace the proline residue in this motif to modulate the preference of C$^\gamma$-ring pucker and PPII forming propensity. Besides the preference of ring puckers for these derivatives, the hydrophobicity and size of their substituting groups can also allow the analysis of consequences of induced hydrophobic and steric effects on the binding affinity of the peptide for the WW domain. On the basis of a previous study,[27] we chose a proline-rich peptide Myt1-T412 as the parent peptide and altered the proline residue in its pT-P motif to generate a series of peptide ligands. We prepared a modified version of the Myt1-T412 peptide, PPA(pT)PPGY (Myt1-Pro), and its six variants, designated Myt1-Hyp, Myt1-Flp, Myt1-Mop, Myt1-hyp, Myt1-flp, and Myt1-mop (Table 1). These peptides were used in the ITC binding assay, with the C-terminal Tyr residue being used for the determination of concentration. Three 5(6)-carboxyfluorescein-labeled peptides, Flu-Myt1-Pro, Flu-Myt1-Flp, and Flu-Myt1-flp, were also synthesized for the fluorescence anisotropy binding assay. In addition, unlabeled and ^{15}N-labeled WW domains were prepared using an *E. coli* expression system. The ^{15}N-labeled protein was used for chemical shift perturbation measurements. Two extra residues were attached to the WW domain because it was expressed as a GST-conjugated protein.

Table 1. Sequences of the Pin1 WW Domain and the Peptides Used in This Study

protein/peptide	sequence[a]
Pin1 WW domain	GS-KLPPGWEKRMSRSSGRVYYFNHITNASQWERPSG
Myt1-Pro	Ac-PPApT(Pro)PGY-NH_2
Myt1-Hyp	Ac-PPApT(Hyp)PGY-NH_2
Myt1-hyp	Ac-PPApT(hyp)PGY-NH_2
Myt1-Flp	Ac-PPApT(Flp)PGY-NH_2
Myt1-flp	Ac-PPApT(flp)PGY-NH_2
Myt1-Mop	Ac-PPApT(Mop)PGY-NH_2
Myt1-mop	Ac-PPApT(mop)PGY-NH_2
Flu-Myt1-Pro	5(6)-carboxyfluorescein-PPApT(Pro)P-NH_2
Flu-Myt1-Flp	5(6)-carboxyfluorescein-PPApT(Flp)P-NH_2
Flu-Myt1-flp	5(6)-carboxyfluorescein-PPApT(flp)P-NH_2

[a]Ac indicates an acetylated N-terminus, and NH_2 indicates an amidated C-terminus. Hyp is (2*S*,4*R*)-4-hydroxyproline, Flp (2*S*,4*R*)-4-fluoroproline, Mop (2*S*,4*R*)-4-methoxyproline, hyp (2*S*,4*S*)-4-hydroxyproline, flp (2*S*,4*S*)-4-fluoroproline, and mop (2*S*,4*S*)-4-methoxyproline.

Analysis of the Binding Affinities of Phosphopeptides for the WW Domain. After the preparation of Myt1-T412-derived peptides, their binding affinities for the WW domain were evaluated by performing ITC analysis. ITC analysis indicates that all the peptides show affinities for the WW domain, except for Myt1-flp, which exhibits no binding to the WW domain. As shown in Figure 1 and Table 2, the dissociation constant (K_d) between the WW domain and the Myt1-Pro peptide was found to be 20 μM, similar to the value (15 μM) determined using fluorescence anisotropy in a previous study.[27] Of the analyzed peptides, only Myt1-Flp exhibits a stronger affinity for the WW domain than Myt1-Pro, with a K_d value of 3.3 μM (Figure 1 and Table 2). These results indicate that replacement of the Pro residue in the pT-P motif with Flp in the peptide increases the binding affinity of the peptide by 6-fold. Other peptides, including Myt1-Hyp, Myt1-hyp, Myt1-Mop, and Myt1-mop, have affinities for the WW domain much weaker than that of Myt1-Pro (Figures S4 and S5 of the Supporting Information and Table 2), suggesting that the incorporation of hydroxyl and methoxyl groups into the pyrrolidine ring may significantly disrupt the interaction

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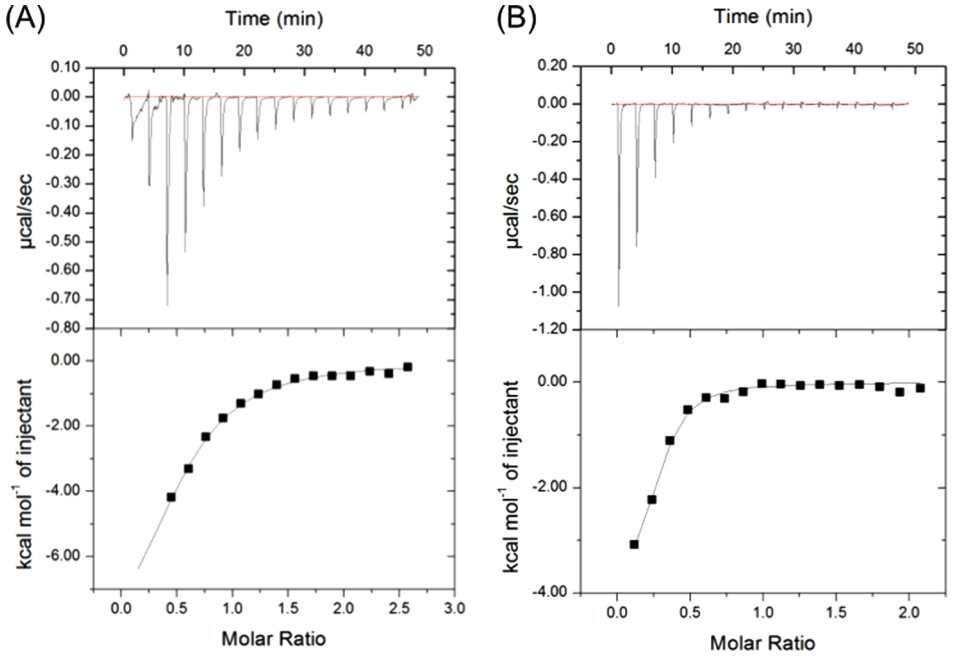

Figure 1. ITC analysis of (A) Myt1-Pro and (B) Myt1-Flp binding to the WW domain at 25 °C. The titrations were conducted in pH 7.1 and 20 mM phosphate buffer. The peaks in the top panel represent the titration of 4 μL of 2.0 mM peptide to 0.2 mM protein. The bottom panel reflects the integrated peak areas normalized to moles of peptide added and corrected for dilution effects, and the solid lines represent the best fits to the binding isotherm using the nonlinear least-squares analysis.

Table 2. Dissociation Constants (K_d) for Pin1 WW Domain−Peptide Complexes Measured by ITC Analysis and Fluorescence Anisotropy

peptide	K_d (μM)[a]
Myt1-Pro	20 ± 3
Myt1-Hyp	>300
Myt1-hyp	117 ± 12
Myt1-Flp	3.3 ± 2.0
Myt1-flp	ND[b]
Myt1-Mop	81 ± 46
Myt1-mop	69 ± 34
Flu-Myt1-Pro	44 ± 7[c]
Flu-Myt1-Flp	15 ± 1[c]
Flu-Myt1-flp	ND[b]

[a]The errors are the standard errors to the fit. [b]Not determined because of very weak binding. [c]Measured by fluorescence anisotropy.

between the ligand and the WW domain. In contrast to Flp, flp results in the complete loss of ligand affinity for the WW domain (Figure S6 of the Supporting Information), indicating that the substitution position plays a crucial role in affecting the affinity for the WW domain.

To confirm the results of ITC analysis, fluorescence anisotropy binding assay was performed to measure the affinity between the WW domain and the three N-terminal fluorescein-labeled peptides, Flu-Myt1-Pro, Flu-Myt1-Flp, and Flu-Myt1-flp. Results of fluorescence anisotropy show that the K_d value is 44 μM for Flu-Myt1-Pro and 15 μM for Flu-Myt1-Flp, whereas the affinity of Flu-Myt1-flp for the WW domain is too weak to be determined (Figure 2 and Table 2). The K_d value of Flu-Myt1-Pro is higher than the value (15 μM) previously measured using fluorescence anisotropy for an N-terminal rhodamine-labeled peptide,[27] which was expected due to different labeled peptides. Fluorescence anisotropy measurements indicate that the 4*R*-fluoroproline (Flp) substituent increases the affinity of Flu-Myt1-Flp compared with that of the intact peptide, while the 4*S*-fluoroproline (flp) substituent causes Flu-Myt1-flp to lose its affinity for the WW domain, concurring with the results of ITC analyses.

Structural analysis of the Pin1−phosphopeptide complex has shown that the binding site of the WW domain is relatively hydrophobic and that the proline following the phosphorylated residue of the peptide mainly interacts with Tyr23 and Trp34 of the WW domain.[27] Thus, a hydrophilic moiety or a bulky substituent on the pyrrolidine ring may prevent the binding of phosphopeptides to the WW domain. Low affinities of Myt1-Hyp and Myt1-hyp observed in this study indicate that the polar hydroxyl group of Hyp or hyp destroys the interaction between the peptide and the WW domain, while the bulky methoxyl group of Mop or mop significantly weakens the affinities of Myt1-Mop and Myt1-mop for the WW domain. In particular, the -OH group exerts a stronger unfavorable effect than the -OMe group, suggesting that hydrophobicity is more critical than steric conflicts in contributing to the binding of peptides to the WW domain. Furthermore, the C^{γ} pucker preference may not be the key element that affects the binding affinity because a biased C^{γ}-*endo* or -*exo* pucker does not have a distinct impact on binding. Surprisingly, the Flp-containing peptide can bind to the WW domain more tightly than the Pro-containing peptide; however, the flp-containing peptide cannot bind to the WW domain. Because C−F is highly hydrophobic and a fluorine atom is smaller than a methoxyl group, the steric strains of methoxyproline and the polar effects of hydroxyproline cannot apply to interpret the observation on fluoroproline-substituted peptides. The results show that the fluorine atom in the 4*R* or 4*S* position exhibits a different effect on the interactions between the WW domain and fluoroproline-containing peptides, and that the 4*R* configuration of Flp is much more favorable within the binding interface. The

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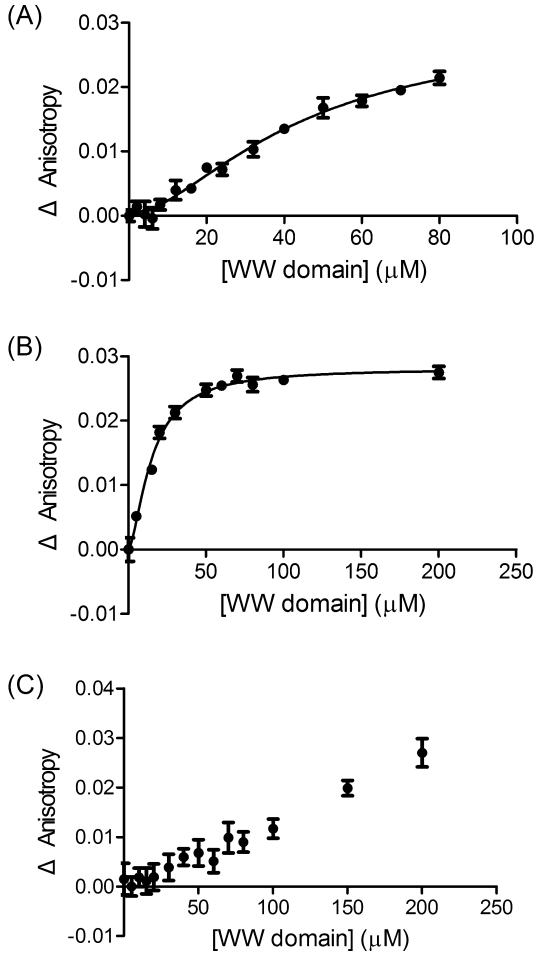

Figure 2. Fluorescence anisotropy binding curves between the WW domain and (A) Flu-Myt1-Pro, (B) Flu-Myt1-Flp, and (C) Flu-Myt1-flp at approximately 25 °C. The measurements were performed in pH 7.1 and 20 mM phosphate buffer. The solid lines represent the best fit to a single-ligand binding model.

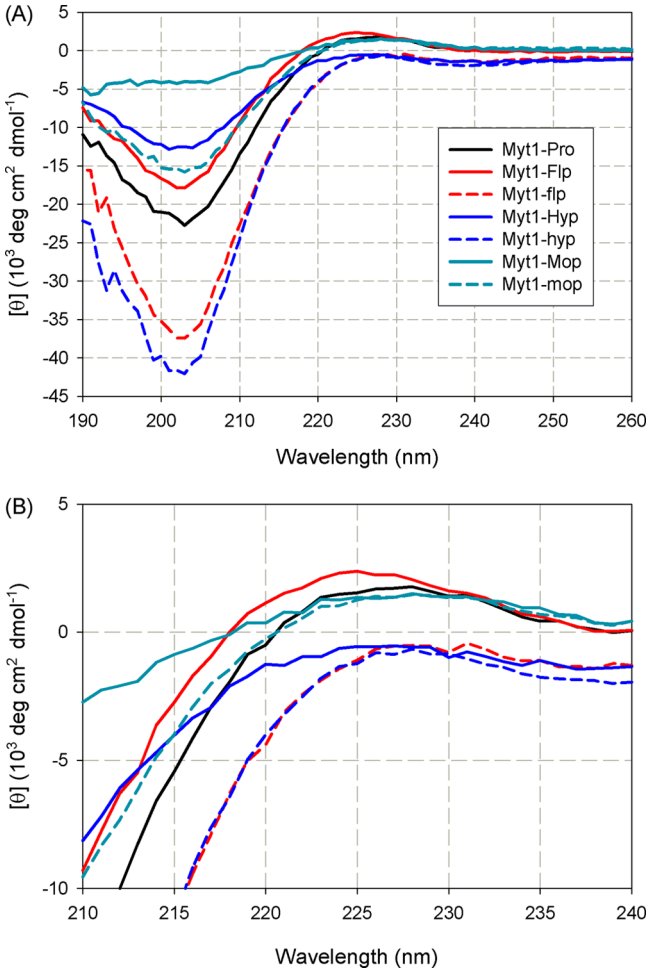

Figure 3. Far-UV CD spectra of the phosphopeptide solutions: (A) full spectra from 190 to 260 nm and (B) comparison of the maximal positive bands. All the measurements were conducted in pH 7.1 and 20 mM phosphate buffer at 25 °C.

opposite effects of Flp and flp on the binding of phosphopeptides to the WW domain also suggest that the incorporation of a fluorine atom into the 4*R* position of proline may induce a relatively suitable conformation to facilitate the binding.

Correlation between Binding Affinity and Peptide Structure. To examine the structures formed by these short phosphopeptides, we also conducted CD measurements on the peptide solutions. As shown in Figure 3, all the peptides form a PPII-like structure with a positive band between 220 and 230 nm and a negative band at approximately 200 nm. Compared to the CD spectrum of Myt1-Pro, all the peptides except Myt1-Flp exhibit a weaker characteristic CD signal of PPII helices. The peptides containing 4*S*-substituted proline derivatives were expected to have a low PPII content because these derivatives prefer a C^{γ}-*endo* pucker and a *cis* prolyl bond.[28,29] However, unlike the previous reports that Hyp- and Mop-containing peptides increased PPII contents because Hyp and Mop prefer a C^{γ}-*exo* pucker and a *trans* peptide bond,[28,29] we observed that Myt1-Hyp and Myt1-Mop have a PPII forming propensity lower than that of Myt1-Pro. This may be because the substitution site is next to a phosphothreonine and the hydroxyl or methoxyl group may interact with the phosphate group, thus making the peptide form a less PPII-like structure. In contrast, Myt1-Flp shows a higher PPII content in solution as expected.

By examining the affinities of these peptides for the WW domain and their PPII contents in solution, we found that the peptide forming a more PPII-like structure could bind to the WW domain more tightly. Of the peptides containing proline derivatives, Myt1-Flp forms the most intense PPII structure and has the lowest K_d value. Previous studies suggested that phosphorylation of the RNA polymerase II C-terminal domain (CTD) conferred it with the PPII conformation and promoted its binding to the WW domain.[26,27] Our results also support the argument that the PPII conformation is a key element required for the strong affinity of the peptide for the WW domain.

Chemical Shift Perturbation Measurements and Molecular Modeling To Reveal the Interaction between Trp34 of the Pin1 WW Domain and the Phosphopeptides. According to the X-crystal structure of the Pin1−CTD complex, the CTD peptide binds to the WW domain, with the phosphoserine of its C-terminal pS-P motif interacting with Ser16 and Arg17 residues of the WW domain and the proline in that motif interacting with Tyr23 and Trp34 residues of the WW domain.[27] This proline residue adopts a C^{γ}-*endo* pucker and *trans* prolyl peptide bond in the crystal structure. Because we used the peptide ligands based on Myt1-T412 [PPA(pT)-

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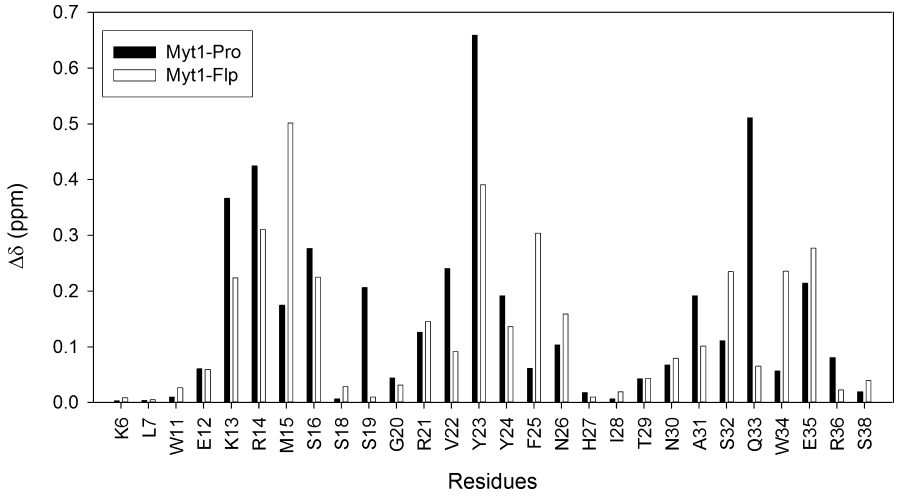

Figure 4. Weighted average of (^{1}H and ^{15}N) chemical shift perturbation of residues in the Pin1 WW domain upon formation of a complex with Myt1-Pro or Myt1-Flp.

PP], which is different from that in the crystal structure, we applied ^{1}H−^{15}N HSQC spectra to measure chemical shift perturbations to determine whether our peptide also interacts with the WW domain in the same region as that in the Pin1−CTD complex. The measurements were conducted using solutions containing only the WW domain and various ratios of the WW domain and peptide ligands. The peptides Myt1-Pro and Myt1-Flp were used in the experiments because they exhibit a high affinity for the WW domain. The HSQC spectra of the WW domain and the phosphopeptide−WW domain mixtures are shown in the Supporting Information (Figures S7−S9). As shown in Figure 4, the chemical shifts of the ^{15}N-labeled WW domain display a similar perturbation profile upon the addition of Myt1-Pro or Myt1-Flp, indicating that both the peptides bind to the WW domain in the same region. As shown in Figure 4, the ^{1}H and ^{15}N chemical shifts of Tyr23 and Trp34 are significantly perturbed, suggesting that the proline residue after phosphothreonine in the Myt1-Pro and Myt-Flp peptides strongly interacts with these two residues as observed in the WW domain−CTD complex.

The studies by Noel and co-workers also showed that Trp34 is more crucial than Tyr23 in this binding because mutating Trp34 dramatically decreases the affinity of the WW domain for its ligands.[27] This suggests that a strong interaction between the side chain of Trp34 and the pyrrolidine ring following the phosphorylated Ser or Thr would lead to a tight binding. Accordingly, the Flp residue in Myt1-Flp may strongly interact with Trp34 within the binding interface to strengthen the binding interaction between Myt1-Flp and the WW domain. To obtain further insights into the interactions between the WW domain and the fluoroproline-containing peptides, we used a molecular modeling approach. On the basis of the reported crystal structure of the Pin1−CTD complex (PDB entry 1F8A), we modified the ligand into our designed peptide and conducted energy minimization in the presence of water by using the Discovery Studio program.

In the crystal structure of the Pin1−CTD complex, the proline residue that follows phosphoserine in CTD peptide and interacts with Trp34 adopts a C^{γ}-*endo* pucker,[27] suggesting that this conformation could be critical for the interaction between the pT/pS-P motif and the WW domain. Accordingly, when we modified this proline into fluoroproline for molecular modeling, we added only a fluorine atom to the 4*R* or 4*S* position of proline without altering its pucker configuration as the initial structure. Our purpose was to examine how the fluoro group would affect the relative orientation and distance between the pyrrolidine ring and the indole ring of Trp34, if the proline ring maintains a C^{γ}-*endo* pucker regardless of modification. The modeling method used is not able to predict how the proline ring pucker preferences themselves might be altered by the modifications. We expected that the pucker should maintain an *endo* conformation without dramatic changes during molecular modeling. As shown in Figure 5, the energy-minimized structures indicate that the Flp and flp residues both adopt a C^{γ}-*endo* pucker in the complexes. According to the modeled structure shown in Figure 5A, the distance between the C^{γ}H group of proline and the indole ring of Trp34 is only 3.18 Å in the energy-minimized structure of the WW domain−Myt1-Pro

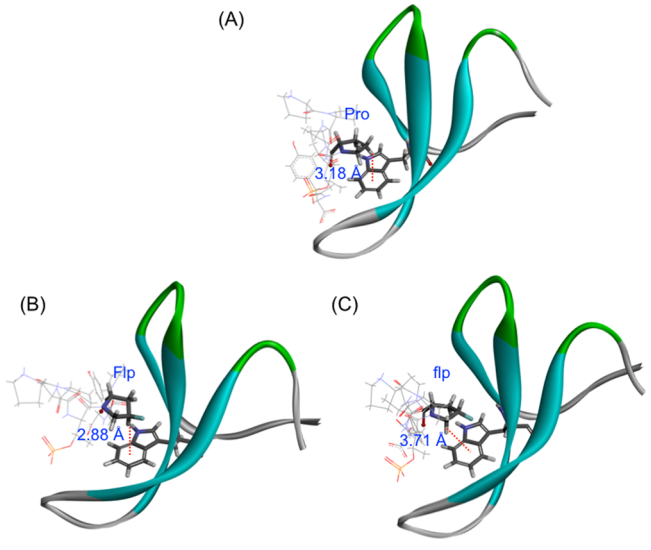

Figure 5. Energy-minimized structures of (A) the WW domain−Myt1-Pro complex, (B) the WW domain−Myt1-Flp complex, and (C) the WW domain−Myt1-flp complex. The shortest distances between the pyrrolidine ring (C^{γ}H in Pro and Flp and C^{δ}H in flp) and the indole ring (Trp34) are shown in blue. The modeling was conducted in the presence of water molecules using PDB entry 1F8A with the Discovery Studio program.

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complex, indicating a C−H···π interaction between them. Such an interaction might mainly contribute to the binding affinity, and similar interacting patterns were also observed in other protein−peptide complexes (e.g., SH3 domains) in which C^{α} or C^{δ} protons interact with an aromatic ring.[39−43] Similar to the energy-minimized structure of the WW domain−Myt1-Pro complex, the energy-minimized structure of the WW domain−Myt1-Flp complex also shows that the distance between the $C^{\gamma}H$ group of Flp and the indole ring of Trp34 is only 2.88 Å (Figure 5B). The shorter distance suggests that the C−H···π interaction of the Flp−Trp pair is stronger than that of the Pro−Trp pair. This can be explained by the fact that the fluoro group on the C^{γ} atom makes the adjacent $C^{\gamma}H$ group more electron-deficient and more positive to enhance the C−H···π interaction.

In contrast, the energy-minimized structure of the WW domain−Myt1-flp complex (Figure 5C) indicates that the $C^{\gamma}H$ group of flp is away from the indole ring of Trp34, which is different from those observed in the other two complexes. In this structure, the shortest distance is 3.71 Å between the $C^{\delta}H$ group of flp and the indole ring of Trp34, indicating that the C−H···π interaction of the flp−Trp pair if any is much weaker than that of the other two pairs. We observed that the relative orientation of flp to Trp is shifted from that of Pro to Trp or that of Flp to Trp in the modeled structures, further suggesting that flp may not fit into an appropriate conformation to generate a favorable C−H···π interaction. In particular, with a C^{γ}-*endo* pucker the fluorine atom of flp points toward the indole ring of Trp34 and may induce a strong electron repulsion to prohibit the binding of Myt1-flp to the WW domain. The modeling results are consistent with our experimental observations that Myt1-Flp has a stronger affinity for the WW domain than Myt1-Pro and that Myt1-flp cannot bind to the WW domain. The results also imply that the C−H···π interaction between pyrrolidine and indole rings is a major contributor to the binding between the peptides and the WW domain.

More interestingly, the CD measurements suggest that the more PPII-like structure of Myt1-Flp may confer it with a higher affinity for the WW domain. However, the energy-minimized structures indicate that Pro, Flp, and flp all adopt a C^{γ}-*endo* pucker that is not favored in a stable PPII helix. Thereby, we propose that a more PPII-like helix of Myt1-Flp could access the binding site of the WW domain more easily. Upon entering the binding pocket, the Flp residue following pT would subsequently adopt a more stable and appropriate conformation to generate a strong C−H···π interaction with Trp34. The contrasting effects of Flp and flp on the phosphopeptide−WW domain interaction observed in this study are consistent with those observed in a previous study by Déprez and co-workers who used dipeptides and NMR measurements to show that Flp increases the binding affinity for the WW domain compared with that of flp.[25] Here we combined various biophysical approaches, including ITC, fluorescence anisotropy, NMR, and molecular modeling, to demonstrate that the strong C−H···π interaction induced by Flp might be the key factor for enhancing the affinity of the phosphopeptide for the WW domain of Pin1.

CONCLUSION

In the study presented here, we used 4-substituted proline derivatives to design the peptide ligands for the Pin1 WW domain and measured their affinities by performing ITC and fluorescence anisotropy assays. Although these proline derivatives favor different ring puckers depending on the substituting group, the puckering effects do not exert an important impact on the ligand affinity for the WW domain. Instead, the hydrophobic and steric properties of these substituting groups are more important for affecting the binding than the pucker conformation. The propensity of forming PPII structure in solution also plays a critical role in affecting the binding affinity of the peptide for the WW domain. Moreover, the C−H···π interaction between the pyrrolidine ring (Pro) and the indole ring (Trp) makes a key contribution to the binding process. For Flp, it possesses a hydrophobic C−F moiety and its fluorine atom causes only minimal steric strains and thus can well fit into the binding pocket of the WW domain. In addition, the high electronegativity of fluorine induces a relatively strong C−H···π interaction between Flp and Trp, making Flp an ideal substituent of Pro to serve as a better ligand for the WW domain. Our findings show that a subtle change on the pyrrolidine side chain of proline could dramatically affect the interaction and the affinity of phosphorylated peptides for the WW domain, which may be applied in the design of inhibitors of Pin1. In conclusion, our study provides a new approach to designing peptide-based ligands or inhibitors of proteins by using 4-substituted proline derivatives.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00880.

Measured mass data, HPLC chromatograms for the phosphopeptides, part of the ITC analyses, and $^{1}H-^{15}N$ HSQC spectra of the WW domain (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PPIase, peptidyl-prolyl isomerase; Pin1, peptidyl-prolyl *cis*/*trans* isomerase NIMA-interacting 1; WW domain, human Pin1 WW domain; CD, circular dichroism; ITC, isothermal titration calorimetry; Fmoc, *N*-9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tet-

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ramethyluronium hexafluorophosphate; HSQC, heteronuclear single-quantum coherence; PPII, polyproline II helix.

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