**p13SUC1 and the WW Domain of PIN1 Bind to the Same Phosphothreonine-Proline Epitope**

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The WW domain of the human PIN1 and p13SUC1, a subunit of the cyclin-dependent kinase complex, were previously shown to be involved in the regulation of the cyclin-dependent kinase complex activity at the entry into mitosis, by an unresolved molecular mechanism. We report here experimental evidence for the direct interaction of p13SUC1 with a model CDC25 peptide, dependent on the phosphorylation state of its threonine. Chemical shift perturbation of backbone $^1$H$_N$, $^{15}$N, and $^{13}$C$_\alpha$ resonances during NMR titration experiments allows accurate identification of the binding site, primarily localized around the anion-binding site, occupied in the crystal structure of the homologous p9CKSH$_2$ by a sulfate molecule. The epitope recognized by p13SUC1 includes the proline at position +1 of the phosphothreonine, as shown by the decrease in affinity for a mutated CDC25 phosphopeptide, containing an alanine/proline substitution. No direct interaction between the PIN1 WW domain or its catalytic proline cis/trans-isomerase domain and p13SUC1 was detected, but our study showed that *in vitro* the WW domain of the human PIN1 antagonizes the binding of the p13SUC1 to the CDC25 phosphopeptide, by binding to the same phosphoepitope. We thus propose that the full cyclin-dependent kinase complex stimulates the phosphorylation of CDC25 through binding of its p13SUC1 module to the phosphoepitope of the substrate and that the reported WW antagonism of p13SUC1-stimulated CDC25 phosphorylation is caused by competitive binding of both protein modules to the same phosphoepitope.

Conserved Ser/Thr kinase complexes drive progression through the different cell cycle phases in eukaryotic cells. The complex consists of a regulatory subunit, the cyclin, and a catalytic subunit, the cyclin-dependent protein kinase (CDK). This latter specifically recognizes the serine/threonine-proline motif, and a structural basis for the proline preference has been recently described (1). A complex interplay of phosphorylation and dephosphorylation by and of the complex regulates tightly the cell cycle; at the G$_2$/M transition, for example, the activation of the complex requires dephosphorylation of the CDK Thr-14 and Tyr-15 residues by the phosphatase CDC25. CDC25 is highly phosphorylated at mitosis, in part by the CDK complex that carries out the up-regulation of CDC25 activity. The hyperphosphorylated CDC25 activates in turn the CDK complex, creating a positive feedback loop.

The kinase complex contains, in addition to the cyclin and the CDK, a small essential regulatory protein, called CKS (cyclin-dependent kinase subunit), whose function is not precisely known. Depletion and overexpression of CKS caused a G$_2$ delay or abolished entry into mitosis, and in the latter case, accumulation of inactive kinase molecules phosphorylated on Tyr-15 (2, 3). Moreover, *in vitro* CKS enhances the phosphorylation of the CDC25, even though the kinase activity of the CDK complex is not directly modified by the CKS binding, suggesting a role for CKS in substrate recognition (4). A similar stimulation of phosphorylation of a CDK substrate by CKS was demonstrated in the case of the CDC27 substrate in the proteasome (5). Furthermore, the CKS from *Schizosaccharomyces pombe*, commonly called the p13SUC1 protein, can bind the proteasome in a phosphorylation-dependent way (6). This evidence leads to a commonly accepted picture where the CKS subunit of the CDK complex targets the activated complex (7) to specific phosphoproteins, such as the CDC25 phosphatase. The presence of a conserved anion-binding site at the surface of CKS molecule, occupied in the crystal structure of human p9CKSH$_2$ by a sulfate molecule (8, 9), gives some structural basis to this hypothesis, although no structural data on CKS with a *bona fide* substrate have been reported.

PIN1 is another essential regulator at mitotic entry that binds mitosis-specific phosphoproteins such as CDC25 (10–14). The human PIN1 protein contains two domains, a 100-residue prolyl cis/trans-isomerase C-terminal catalytic domain and a small N-terminal WW domain (15). The WW domain of the human PIN1 is responsible for the binding to the Thr(P)/Ser(P)-proline motifs (16). The catalytic domain shows specificity for the Thr(P)/Ser(P)-Pro bond. Both site-specific catalytic activity and binding are essential for PIN1 biological activity (16, 17). A functional interplay between the PIN1 protein and the CDK complex was recently demonstrated, as PIN1 completely abolishes the stimulation of the CDK-mediated phosphorylation of CDC25 by CKS (4). Intriguingly, only the WW domain seems to be involved in this antagonistic action (4). Similar to the CKS protein, PIN1 does not affect the CDK
kinase activity directly, at least in vitro, suggesting that competition between CKS and the WW domain for the phosphorylated substrate might be at the origin of the antagonistic action of PIN1.

NMR spectroscopy is most suitable to investigate potential molecular interactions and can lead to accurate values of interaction constants but also to mapping of the residues implicated in the interaction (both on the protein target and the peptidic ligand), kinetic parameters related to interaction and/or catalysis, and eventually a structural model of the interaction complex. We report here the results of our NMR study to determine whether the CKS protein indeed binds phosphorylated substrates, such as a model CDC25 peptide containing a Thr(P). We found a phosphorylation-dependent binding of p13SCUC1 to the peptide substrate, and we mapped the interaction site by a chemical shift perturbation method. The importance of the proline residue at position +1 following the Thr(P), as in the threonine-proline motif corresponding to the minimal CDK recognition site, was demonstrated. The affinity of p13SCUC1 for a phosphopeptide containing an alanine/proline substitution at position +1 following the Thr(P) was indeed significantly decreased. Finally, we checked whether the antagonism between the WW domain of PIN1 and CKS for the phosphorylation of CDC25 could be due to direct interaction between both proteins or, alternatively, to their competition for binding the phosphorylated substrate. No direct interaction was detected, confirming that the competition for the same substrate is the underlying molecular mechanism of the observed antagonism.

EXPERIMENTAL PROCEDURES

Protein Expression, Peptide Synthesis, and Purifications—p13SCUC1 (Swissprot accession number P08468) and p13PA90 were expressed in Escherichia coli BL21(DE3) using the T7 promoter-based vector pRK172 (18). p13SCUC1 mutagenesis will be described elsewhere.

15N,13C-Labeled proteins were prepared by growing cells in M9 minimal medium (19) with 15NH4Cl (1 g l−1) and 13C glucose (2 g l−1) (Cambridge Isotope Laboratories, Cambridge, MA) as the sole nitrogen and carbon sources, respectively. P13PA90 was purified by anion exchange with a Q-HyperD column (Biosepra, Marlborough, MA) equilibrated in 50 mM Tris-HCl, pH 8.0, followed by gel filtration with a Superdex 200 column (Amersham Pharmacia Biotech) in Tris-HCl, 50 mM, pH 8.0, and finally by reverse phase chromatography with a Poros 50R1 column (Perseptive Biosystems, Framingham, MA) equilibrated in 0.1% trifluoroacetic acid and developed with an acetonitrile gradient. Synthesis of phosphopeptides is as described (20). The 10-amino acid phospho-CDC25 peptide used in this study is derived from the conserved Thr(P)-Pro site at Thr-48 of CDC25 of Xenopus laevis (Swissprot accession number P30309); the exact sequence is QPLpTPVTDL. The human WW domain from PIN1 (Swissprot accession number Q13526) was obtained by chemical synthesis and purified by reverse phase with a C18 Hyperprep column.

NMR Spectroscopy—All the NMR experiments were performed in a buffer of 50 mM dextrose Tris-HCl, pH 6.3 (Cambridge Isotope Laboratories), 100 mM NaCl, and 1 mM DTT. The spectra were recorded at 20 °C on a Bruker 600-MHz DMX spectrometer (Bruker, Karlsruhe, Germany). Sequential backbone resonance assignment of p13PA90 was achieved using the following three pairs of triple resonance (three-dimensional) experiments: HNCA/HNCO/CA (Biosepra, Marlborough, MA) and 15N-edited HSQC NOESY/15N-edited HSQC TOCSY as will be described elsewhere.2 Increasing amounts of unlabelled synthetic peptide of sequence EQPLpTPVTDL (phospho-CDC25 peptide) or EQPLpTPVTDL (Ala/Pro-substituted phospho-CDC25 peptide) were added to a [15N,13C]p13PA90 sample. Final concentrations were successively 0.83/0.22, 0.82/0.40, 0.78/0.80, 0.70/1.7, 0.66/2.2, and 0.66/3.5 mM for the p13PA90/phospho-CDC25 peptide sample and 0.66/3.3 mM for the p13PA90/Ala/Pro-substituted phospho-CDC25 peptide sample. 

Antagonism of p13 and WW Domain

Interaction of p13PA90 with the phospho-CDC25 peptide (A and B) or Ala/Pro-substituted phospho-CDC25 peptide (C and D). Overlaid sections of $^{15}$N-HSQC spectra acquired on p13PA90, before (in black contours) and after addition of 0.25 (orange); 0.5 (red), 0.75 (pink), 1 (violet); 2 (dark blue); 3 (light blue) and 5 (green) molar equivalent of phospho-CDC25 peptide (A and B) or Ala/Pro-substituted phospho-CDC25 peptide (C and D). Resonances are labeled. B–D, a gradual chemical shift perturbation of residue Gly-81 (B and D) or residue Trp-82 (C) can be observed, due to fast exchange between bound and unbound form of p13PA90 on the NMR time scale. A, an intermediate exchange leads to disappearance of residue Trp-82 signal.

The interactions are mainly localized in the β-sheet of p13PA90. Large chemical shift modifications were observed for the conserved surface residues forming the sulfate-binding site (8, 9), which are residues Arg-30, Arg-39, Gln-78, Trp-82, and Arg-99 in p13$^{SUC1}$. These residues are most likely in direct contact with the phosphorylated CDC25 peptide. The largest perturbations were observed in the loop connecting helix-α3 and the β3-strand of the β-sheet (Fig. 2A and Fig. 3). The other smaller resonance perturbations corresponded to neighboring residues, with similar small chemical shift perturbation of residues located in the α3-helix (Glu-68, Glu-69, Glu-70, Arg-72, and Gly-73). As a control, we also recorded an HSQC spectra of the p13$^{SUC1}$ wild-type protein in the presence of an excess of phospho-CDC25 peptide, and we found identical perturbations as those of the mutant protein for all the resonances that could be observed (data not shown). The observed interaction is specific for the phosphorylated peptide, as no modification of the p13PA90 resonances was observed upon addition of the non-phosphorylated control peptide (data not shown).

However, the addition of inorganic phosphate did cause chemical shift perturbation of the same residues implicated in the phospho-peptide binding. The perturbations were, however, less important, and a binding constant of 40 mM was estimated. This indicates that in addition to the phosphate group on the threonine, other contacts were involved in the binding of the phospho-peptide by the p13PA90.

The CKS Protein Is Binding a Phosphothreonine within a Threonine-Proline CDK Epitope—As the motif threonine-proline is the minimal recognition site of the CDK, we wanted to test if the CKS had a similar specificity for the proline at position +1. The NMR titration experiment was repeated with a modified phospho-CDC25 peptide that contained an alanine instead of a proline at the position +1 following the Thr(P). The residues that showed major changes upon binding of the original phospho-CDC25 peptide (Gln-78, Ser-79, and Trp-82) were also affected by addition of the Pro/Ala-substituted phospho-CDC25 peptide, but at an equivalent molar ratio of peptide to p13PA90 protein, the chemical shift perturbations observed upon addition of Pro/Ala-substituted phospho-CDC25 peptide were far inferior to the equivalent results obtained with the phospho-CDC25 peptide (Fig. 1 and Fig. 2B). The dissociation constant $K_d$ was estimated by a titration experiment, based on the chemical shift change observed for 5 residues (Thr-77, Gln-78, Gly-81, Trp-82, and Lys-98) to be 5.4 ± 1 mM, a 6-fold increase compared with the dissociation constant for the phospho-CDC25 peptide (Fig. 2B). The proline residue at position +1 is thus clearly involved in the recognition of the substrate by the p13$^{SUC1}$ protein, as could be expected for an adaptor protein of the CDK complex to its substrate.

Competition of WW and p13$^{SUC1}$ for Binding to a Phosphorylated CDC25 Peptide—We next asked whether p13$^{SUC1}$ and PIN1 interact through the WW and/or the catalytic domain of the latter, affecting potentially the binding of p13$^{SUC1}$ to the phospho-CDC25 peptide. The resonances of p13PA90 in the $^{1}H$-$^{15}$N HSQC were not significantly affected by addition of an equimolar amount of WW domain alone, and no catalytic activity of the prolyl cis/trans-isomerase domain PIN1At from A. thaliana on the Ghu-91–Pro-92 motif of p13PA90 was observed. This precludes a direct interaction between PIN1 and CKS to be at the basis of the functional antagonism between both molecules (4).

The WW domain of PIN1 was previously shown to bind to the phospho-CDC25 peptide (16). This interaction was indeed verified by NMR spectroscopy, and the molecular contacts between the CDC25 peptide and the WW domain agreed with the recent structural data (30) on the complex between PIN1 and a peptide representing a heptad repeat of the RNA polymerase II C-terminal domain of the large subunits.

More importantly, we could follow the molecular competition between PIN1 and CKS by adding the WW domain to a 0.8 mM [15N,13C]p13PA90/0.8 mM phospho-CDC25 sample. After addition of unlabeled WW, resulting in final concentrations of 0.5 mM p13PA90/0.5 mM WW/0.5 mM phospho-CDC25 peptide, we observed a significant decrease in the chemical shift perturbation of the p13PA90 resonances (Fig. 4). The p13PA90 resonances observed in the presence of WW were shifted back at a position roughly equivalent to the ones observed in the sample 0.8 mM p13PA90/0.25 mM phospho-CDC25. This showed that an important fraction of the phospho-CDC25 peptide is unavailable to p13PA90 binding, due to competitive binding of the WW domain.

Addition of the prolyl cis/trans-isomerase catalytic domain PIN1At of A. thaliana to a p13PA90/phospho-CDC25 sample to final concentrations of 0.35 mM p13PA90/0.35 mM PIN1At/1 mM phospho-CDC25 did not result in significant modifications of the HSQC spectra compared with the equivalent spectra of a 1:3 p13PA90/phospho-CDC25 sample without PIN1At, although the phospho-CDC25 peptide was a good substrate for the PIN1At enzyme (data not shown (20)). We therefore conclude that the catalytic domain of PIN1At alone does not affect directly the binding of the p13PA90 to the phospho-CDC25 peptide.

**DISCUSSION**

We have shown by NMR chemical shift perturbation mapping that p13$^{SUC1}$ is able to bind a phosphopeptide corresponding to a fragment of CDC25 centered on Thr-48. As revealed by

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large chemical shift perturbations, the residues involved in the binding correspond primarily to the conserved sulfate-binding site (Arg-30, Gln-78, Trp-82, and Arg-99) reported in an earlier structural study of the p13CKS2 homologue (8, 9). The largest perturbations were observed in the loop connecting the α3-helix and the β3-strand (Thr-77, Gln-78, Ser-79, Leu-80, Gly-81, and Trp-82). The other smaller observed perturbations correspond to neighboring residues in the β-sheet and to residues from helix-α3. The interaction was shown to be dependent on the phosphorylation of the Thr and to involve the Pro at position +1 following the Thr(P). Although the exact motif recognized by the CKS could be even more specific, the Ser/Thr kinase that generates this motif was previously shown to be proline-directed (1).

Based on our results, we propose that the stimulation of the CDC25 phosphorylation by the CDK complex in the presence of CKS (4) is mediated by binding of the CKS to the CDC25 substrate. The estimated dissociation constant of the order of 1 mM is rather low but could be higher when the full protein rather than a peptide is the substrate. A second factor contributing potentially to an increased affinity might be the stabilization of the highly flexible β-sheet of CKS by the binding to the CDK kinase. The affinity of the CKS for the CDK complex is high (18) (the Kd is estimated to be about 100 nM (23)), and most probably the CKS will only interact with its substrate when it is itself bound to the CDK kinase. Finally, if the CKS has to target the CDK complex to its substrate, a high turnover might be necessary, with preference of weak over strong binding constants.

It was recently shown that PIN1 completely abolishes the stimulation of the CDK-mediated phosphorylation of CDC25 by CKS, although neither p13SUC1 nor PIN1 affect directly the CDK activity, at least in vitro (4). We first tested if PIN1 had any interaction with the CKS protein. The conserved EP motif found in the β-hinge of the CKS could indeed be a potential substrate of the catalytic prolyl cis/trans-isomerase domain of PIN1 (11, 12). This β-hinge plays an important role in the CDC25 phosphorylation by the CDK complex in the presence of CKS (4) is mediated by binding of the CKS to the CDC25 substrate. The estimated dissociation constant of the order of 1 mM is rather low but could be higher when the full protein rather than a peptide is the substrate. A second factor contributing potentially to an increased affinity might be the stabilization of the highly flexible β-sheet of CKS by the binding to the CDK kinase. The affinity of the CKS for the CDK complex is high (18) (the Kd is estimated to be about 100 nM (23)), and most probably the CKS will only interact with its substrate when it is itself bound to the CDK kinase. Finally, if the CKS has to target the CDK complex to its substrate, a high turnover might be necessary, with preference of weak over strong binding constants.

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binding of CKS to the CDK (24, 25) and in the stability and folding of the CKS protein (21).2 We did not observe by NMR chemical shift perturbation analysis any significant binding to or modification of p13PA90 by the PIN1At catalytic domain of A. thaliana. The PIN1At enzyme had also no effect on the binding of the phospho-CDC25 peptide by p13PA90, although we observed catalysis of the cis/trans-isomerization of the Thr(P)-Pro bound contained in the phospho-CDC25 peptide (20).

Not only the catalytic domain but also the WW domain of human PIN1 does not interact directly with p13 [81 copper]. However, it does bind to the same Thr(P)-Pro motif as CKS, classifying both of them as proline-directed phosphothreonine-binding modules. This differentiates them from another sequence-specific phosphopeptide-binding protein, the 14-3-3 protein (26, 27), that is also involved in CDC25 binding and regulation of the entry into mitosis (28, 29) but binds an Arg-Ser-X(Ser)-X-Pro motif, where X can be any amino acid (27). The distinction can be further extended to the structural level, with WW and CKS both being mainly β-sheet proteins that use their β-sheet to construct the binding site (16), whereas the anion-binding site on the 14-3-3 protein is located on a surface composed of α-helices (27).

The structure of the protein PIN1 in complex with a phosphoserine-containing peptide shows that the proline is recognized by aromatic residues Tyr-23 and Trp-34 on the WW domain and that the Ser(P) is in direct contact with residues Ser-16 and Arg-17 located in the loop I connecting the strand β1 and β2 (30). The recognition is highly selective but of low affinity, consistent with our results. Recognition of Pro-rich ligand peptides by a different type of WW domain was shown to be similar to that found in SH3 complexes, although both protein modules have different structures (31). That this might result in a molecular competition is indicated by the polyproline region of formin binding interchangeably to the WW or SH3 domain of a formin-binding protein (32, 33). The presented evidence of direct competition between the WW domain of PIN1 and CKS for Thr(P)-Pro-containing peptides could in a very similar way regulate the substrate binding of CKS and hence the CDK activity.

Our data are consistent with a previous biochemical study that strongly suggests that PIN1 could slow down the entry into mitosis by competition with the CKS protein for binding to substrates of the CDK complex (4). In vitro, PIN1 was shown to interact with CDC25 (13, 14), and the overexpression of CKS leading to accumulation of inactive kinase molecules phosphorylated on Tyr-15 indicates a direct interaction between CKS and CDC25 (2, 3). Moreover, recent studies have shown that the PIN1 protein regulates negatively the entry into mitosis (10, 13, 14) and is necessary for the replication checkpoint control (17). Therefore, although the previously described in vitro competition for the same substrate (4) and our direct interaction mapping do not prove of itself that PIN1 and CKS compete in vitro, the combined evidence points toward a balance between both proteins with the level of phosphorylation of mitotic phosphoproteins ensuring a precise control of the timing of entry into mitosis. The role of the prolyl cis/trans-isomerase catalytic domain remains an unclarified point, as we were not able to detect any effect of this domain on binding of phosphorylated substrate by the CKS. The entire PIN1 protein is necessary to perform its essential function in vivo (34) and to ensure the replication checkpoint at mitosis (16, 17), and this point will be the subject of further research.

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