Pin1 modulates the dephosphorylation of the RNA polymerase II C-terminal domain by yeast Fcp1

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Abstract The reversible phosphorylation of serine and threonine residues N-terminal to proline (pSer/Thr–Pro) is an important signaling mechanism in the cell. The pSer/Thr–Pro moiety exists in the two distinct *cis* and *trans* conformations, whose conversion is catalyzed by the peptidyl–prolyl isomerase (PPIase) Pin1. Among others, Pin1 binds to the phosphorylated C-terminal domain (CTD) of the largest subunit of the RNA polymerase II, but the biochemical and functional relevance of this interaction is unknown. Here we confirm that the CTD phosphatase Fcp1 can suppress a Pin1 mutation in yeast. Furthermore, this genetic interaction requires the phosphatase domain as well as the BRCT domain of Fcp1, suggesting a critical role of the Fcp1 localization. Based on these observations, we developed a new in vitro assay to analyze the CTD dephosphorylation by Fcp1 that uses only recombinant proteins and mimics the in vivo situation. This assay allows us to present strong evidence that Pin1 is able to stimulate CTD dephosphorylation by Fcp1 in vitro, and that this stimulation depends on Pin1's PPIase activity. Finally, Pin1 significantly increased the dephosphorylation of the CTD on the Ser[5]–Pro motif, but not on Ser[2]–Pro in yeast, which can be explained with Pin1's substrate specificity. Together, our results indicate a new role for Pin1 in the regulation of CTD phosphorylation and present a further example for prolyl isomerization-dependent protein dephosphorylation.

Key words: CTD; Dephosphorylation; Phosphorylation; Pin1; PPIase; RNA polymerase II

1. Introduction

In contrast to most other peptide bonds, peptidyl–prolyl bonds can exist in the two distinct *cis* and *trans* conformations in peptides and in proteins [1–3]. These two conformations can be converted into each other, a reaction that is catalyzed by peptidyl–prolyl isomerases (PPIases) as originally described by Fischer and coworkers [4,5]. There are two extensively characterized families of conventional PPIases, cyclophilins and FKBPs. Recently, a third family of PPIases has been identified, which can be divided into subfamilies based on their substrate specificity: the parvulin-type and the Pin1-type PPIases [6,7]. The PPIases are ubiquitous enzymes and have been shown to be involved in protein folding, protein translocation through biological membranes, and signal transduction [1,2,8–12].

The highly conserved Pin1-type PPIases are the only PPIases shown to be essential in HeLa cells and in budding yeast [11,13]. Pin1 and its homologues from other organisms, like *Saccharomyces cerevisiae* Ess1/Ptf1 [13,14], *Drosophila* Dodo [15], *Neurospora crassa* Ssp1 [2], and others, consist of an NH_2-terminal WW domain and a COOH-terminal PPIase domain. WW domains, which are characterized by two invariant tryptophans, are present in a variety of signaling and regulatory proteins and were originally identified as protein interaction modules that bind to proline rich regions of their targets [16,17]. The WW domain of Pin1, however, has recently been shown to function as a phosphoprotein-binding module that binds to specific phosphoserine– and phosphothreonine–proline (pSer/Thr–Pro) motifs [18]. Ser/Thr–Pro motifs are targets for proline-directed kinases, and the activity of these kinases is thought to create the binding sites for the WW domain of Pin1 [18]. The PPIase domain of the Pin1-type proteins, which shows some homology to parvulin from *Escherichia coli* [19] and its human homologue Par14 [20,21], exhibits a very distinct substrate specificity. In contrast to parvulin-type PPIases, these proteins specifically isomerize only phosphorylated Ser/Thr–Pro bonds, but not their unphosphorylated counterparts [2,22]. In addition, Pin1 has been shown to inhibit Cdc25c activity and to restore the function of phosphorylated tau, a protein involved in Alzheimer's disease [23,24]. Recently, Pin1 homologues from different plant species have been described [6,7,25]. These proteins do not have an amino-terminal WW domain, but their PPIase domain shows the same substrate specificity as other Pin1-type proteins [7,25]. Thus, the existence and the biological function of phosphorylation-specific PPIases are highly conserved throughout eukaryotic cells.

Until recently, the only enzymes known to be able to distinguish between the *cis* and the *trans* peptidyl–prolyl bond were proteases like chymotrypsin A or subtilisin [1]. These proteases are *trans*-specific, cleaving their substrate only if the preceding Xaa–Pro bond is in the *trans* conformation [1]. New reports, however, have shown that the serine/threonine phosphatase PP2A is also conformation-specific and effectively dephosphorylates only the *trans* pSer/Thr–Pro isomer [12]. In addition, Pin1 is able to accelerate the dephosphorylation of Cdc25c and tau by PP2A [12]. Regarding these results, the phosphorylation-specific prolyl isomerization cata-

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lyzed by Pin1 appears to play an important regulatory role in the dephosphorylation of proteins.

One of the proteins that is able to suppress the lethal phenotype of yeast strains with a temperature-sensitive mutation in the yeast Pin1 homologue Ess1/Ptf1 is the phosphatase Fcp1 [26]. Fcp1 is a specific phosphatase for the C-terminal domain (CTD) of the largest subunit of the RNA polymerase II (RNAP II), Rpb1, and is the only CTD phosphatase known today [27–30]. Fcp1 has been shown to be essential in yeast [31], is needed for global transcription of protein genes [31] and is required for the recycling of RNAP II [30].

Interestingly, an interaction between Pin1 and the phosphorylated form of the CTD has been described by different groups [32,33]. The CTD consists of a seven-amino acid motif with the consensus sequence $Tyr^1Ser^2Pro^3Thr^4Ser^5Pro^6Ser^7$, which is repeated 52 times in humans and 26–27 times in yeast (reviewed in [34]). It is essential for cell viability and plays an important role in the coordination of the various enzymatic steps involved in mRNA formation [35]. The CTD is subject to a complex regulatory mechanism based on various phosphorylations [36]. In vivo, two main forms of RNAP II that differ in their CTD status have been characterized. The IIA form is not phosphorylated on the CTD, is competent to bind to basal promoters of eukaryotic genes and preferentially enters the initiation complex. Shortly after initiation the IIA form is converted into the IIO form by extensive phosphorylation of the CTD on Ser^2 and Ser^5 of the consensus heptapeptide by proline-directed kinases [36,37]. RNAP IIO is found in elongation complexes as well as in pre-mRNA processing events like 5′ capping, polyadenylation, and splicing [37]. Furthermore, different phosphorylation stages in Ser^2 and Ser^5 of the heptapeptide repeat of the CTD seem to occur during the transcriptional process, suggesting the involvement of multiple kinases and phosphatases to modify the CTD [38]. Upon synthesis of a complete mRNA, the CTD must be dephosphorylated, presumably by Fcp1, in order to initiate a new cycle of transcription. However, it has not been shown whether Pin1 affects the catalytic activity of Fcp1 towards the CTD.

Here we show that both the phosphatase domain as well as the BRCT domain of the CTD phosphatase Fcp1 are required to suppress the lethal phenotype of the yeast Pin1 mutant. Based on these observations we designed a new and sensitive in vitro assay that uses only recombinant proteins to analyze the CTD dephosphorylation by Fcp1. Using this assay, we show that Pin1 accelerates the dephosphorylation of the CTD by Fcp1 and that this effect depends on Pin1's PPIase activity. These results are supported by Western blot analysis of lysates from yeast cultures with phospho-specific CTD antibodies, which provide the first in vivo evidence that the CTD dephosphorylation is enhanced by overexpression of Pin1 in yeast cells. Our experiments suggest that Fcp1 is a conformation-specific phosphatase, and thus provide a further example for the use of phosphorylation-specific peptidyl–prolyl *cis*/*trans* isomerization as a regulatory mechanism in the dephosphorylation of proteins.

2. Materials and methods

2.1. Expression of yFcp1 and its mutants in the S. cerevisiae strain YPM2

A DNA fragment encoding the full length Fcp1 protein was generated by PCR using genomic yeast DNA and the primers Fcp1for (5′-GAGA GGATCCT ATG ACC ACA CAA ATA AGG TCT) and Fcp1rev (5′-GAGA CTCGAG AAGCTT CTA ATC ATC CAG CAT ATC CAT). The PCR product of 2.2 kb was sequenced and cloned into the vector pBC100, which allows for the production of HA-tagged proteins in yeast under the control of the GAL1 promoter, as described previously [11]. The Fcp1 mutants Fcp1ΔNLS, Fcp1ΔBRCT, and Fcp1ΔPP were constructed using appropriate restriction sites within the DNA sequence of Fcp1. The reading frames of all deletion mutants were checked by DNA sequencing.

YPM2 cells were grown overnight in YAPD medium at 23°C and then transformed with yFcp1/pBC100 and its mutants using the PEG method. The transformed cells were plated onto minimal medium containing 2% glucose but lacking the amino acid leucine to select for transformants and grown at 23°C for 5–7 days. Colonies were then restreaked at least two times and incubated under the same conditions. For the spotting analysis, single yeast colonies were suspended in TE buffer. The OD_{600} was adjusted to 1, a 10-fold series dilution in TE buffer was made, and 5 μl of each dilution was spotted onto the plates. All plates were made of minimal media without leucine and contained 2% glucose (no induction) or galactose (induction). The plates were incubated for several days at 23°C (permissive conditions) or 30°C (restrictive conditions). As controls, YPM2 cells carrying only the vector pBC100 were used.

The expression of all proteins in YPM2 was verified by Western blotting using an anti-HA antibody (12CA5). YPM2 cells carrying the different constructs were grown at 23°C in minimal media without leucine and a mixture of 1% glucose and 1% galactose to induce protein expression. The cells were harvested and lysed with glassbeads, and 50 μg of the lysates was separated on a 7.5% SDS–PAGE gel and blotted on a nylon membrane.

2.2. Production and purification of proteins

Human Pin1 was produced as described previously [11,12]. Fcp1 and RAP74 proteins were produced and purified as described [43,48]. The plasmid encoding GST-CTD was a gift from Y.J. Wang. The protein was expressed in *E. coli* BL21 and purified as described previously [41,42].

2.3. Dephosphorylation of GST-CTD in vitro

Purified GST-CTD was phosphorylated with [γ-^{32}P]ATP and the kinase cdc2. In general, 25 μg GST-CTD was phosphorylated by 40 U cdc2 (NEB) overnight at room temperature in kination buffer (100 mM KCl, 10 mM $MgCl_2$, 37.5 mM Tris–HCl pH 8.0, 50 μM ATP). GST-CTD(^{32}P) was purified using a PD10 column (Amersham Pharmarcia) equilibrated with Fcp1 reaction buffer [31]. Fractions that contained GST-CTD(^{32}P) but no [γ-^{32}P]ATP were stored at −80°C until use in a dephosphorylation assay.

For the dephosphorylation assay on glutathione beads (Sigma), purified GST-CTD(^{32}P), GST-RAP74(648–735) or GST were preincubated with glutathione beads in dephosphorylation buffer for 1 h at 4°C. The beads were washed, and, if applicable, additional proteins like Pin1 or its mutants were added and preincubated for another 15 min. Finally, the assay was started by the addition of His_{10}-yFcp1. At each time point the reaction was mixed, 10 μl aliquots were removed and directly added to SDS–PAGE loading buffer to stop the reaction. All samples were kept on dry ice and, upon completion of the time course, directly loaded onto 15% SDS–PAGE gels for analysis. GST-CTD(^{32}P) and the released ^{32}P from the phosphatase reaction, which runs with the loading dye front, were visualized with X-ray film. The films were scanned and analyzed using the NIH image software.

2.4. Dephosphorylation of the CTD in vivo

The *S. cerevisiae* strain YPM2 was transformed with pBC100-based constructs that allow for a galactose-induced expression of HA-tagged full length Pin1, the WW domain and the PPIase domain, as described before [12]. The expression of all proteins upon galactose induction was verified by Western blotting. Liquid cultures of YPM2 containing the various expression constructs or the empty vector pBC100 were grown overnight at room temperature in minimal medium without leucine and a glucose/galactose mixture as carbon source. The zero time point sample was withdrawn at an OD_{600} of 0.8, and the remaining culture was quickly adjusted to 37°C as described [39]. The yeast cultures were incubated at 37°C and samples were withdrawn at the indicated time points. All culture samples were immediately centrifuged, washed once with ice-cold H_2O, and frozen in liquid nitrogen.

The yeast pellets were resuspended in lysis buffer as described by Patturajan et al. [40] and adjusted to the same OD_{600}. SDS–PAGE loading buffer and glassbeads (Sigma) were added, and the yeast cells were lysed as described [40]. After recovering the yeast suspension and boiling for 10 min, 15 μl of the supernatant was loaded onto denaturing 7.5% acrylamide gels. The gels were blotted and the phosphorylation status of the CTD was analyzed using the phospho-specific antibodies H5 and H14 [40]. Coomassie staining of the blot and using the anti-tubulin antibody YOL1/34 (Abcam) verified the loading of equal amounts of yeast lysates on the acrylamide gels.

3. Results and discussion

3.1. Both the phosphatase domain and the BRCT domain are required for Fcp1 to rescue an Ess1/Ptf1 mutant in yeast

Fcp1 overexpression has been shown to rescue the lethal phenotype of various temperature-sensitive mutations in Ess1/Ptf1 at the restrictive temperature [26]. However, it is not known whether the different domains of Fcp1, namely

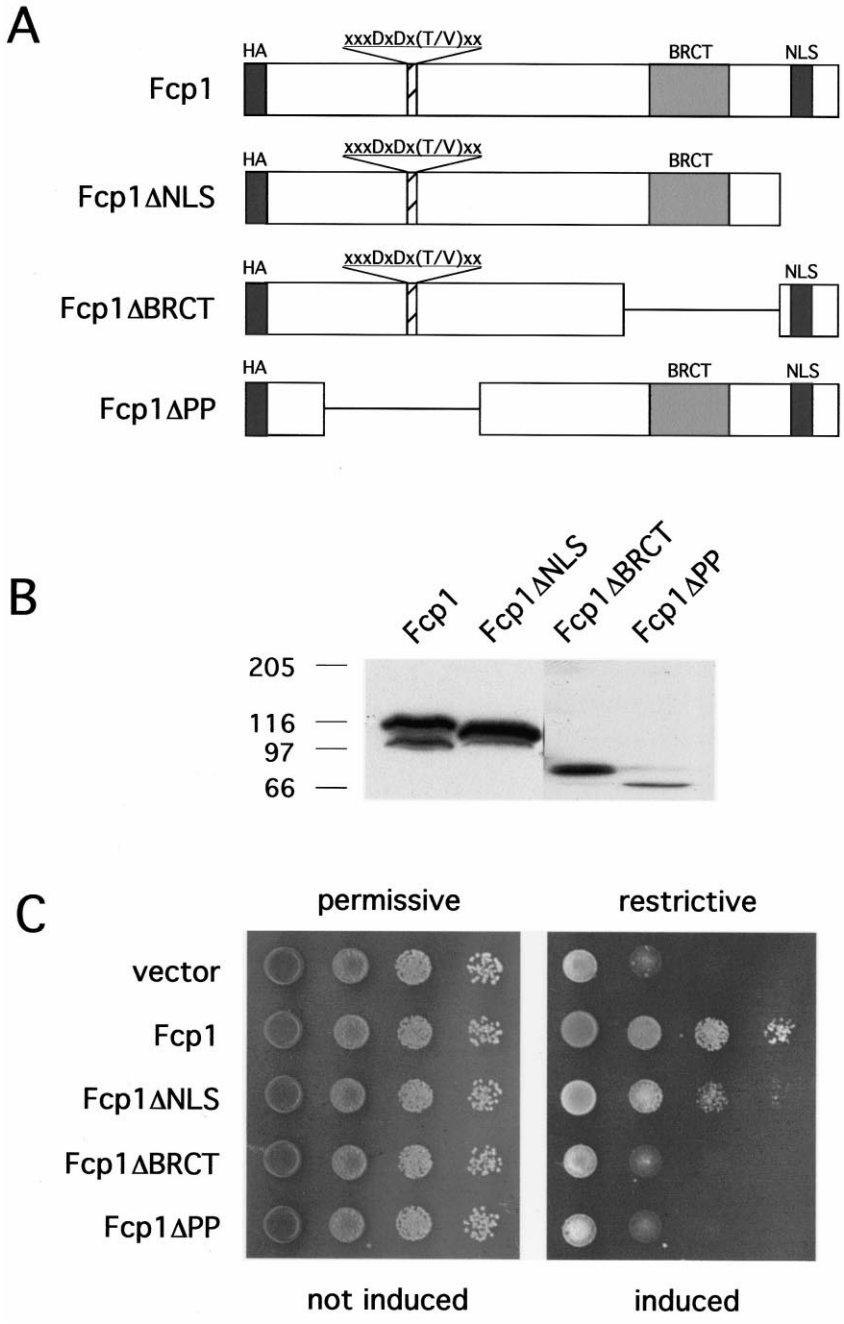

Fig. 1. The phosphatase and the BRCT domain of Fcp1 are required to functional rescue the Ess1/Ptf1 mutation in the yeast strain YPM2. A: Scheme of full length and various deletion mutants of Fcp1. B: Expression of Fcp1 and its deletion mutants in the yeast strain YPM2. YPM2 cells transformed with pBC100-based constructs of the full length and the mutated proteins were grown in glucose/galactose media at room temperature. Expression of HA-tagged proteins is induced under these conditions. The expressed proteins were then detected by Western blotting using an anti-HA antibody. C: Functional rescue of the yeast Ess1/Ptf1 mutation by Fcp1 and its mutants. YPM2 cells transformed with the Fcp1/pBC100, Fcp1ΔNLS/pBC100, Fcp1ΔBRCT/pBC100, and Fcp1ΔPP/pBC100 expression constructs were spotted in a series 10× dilution onto agar plates. The plates were incubated at 23°C (permissive) or 30°C (restrictive). Growth of the cells indicates the complementation of the YPM2 mutation. As control a strain that has only the empty vector pBC100 was used.

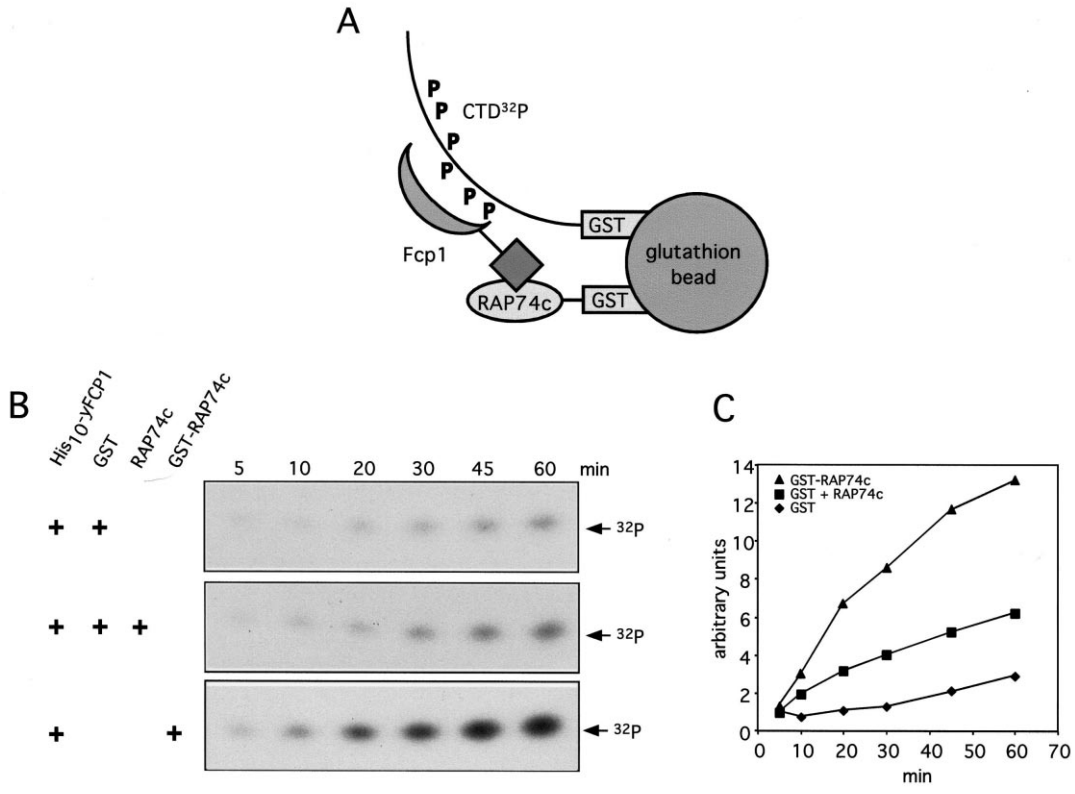

Fig. 2. Development of the novel in vitro matrix-based CTD dephosphorylation assay with Fcp1. A: Schematic presentation of the matrix-based CTD dephosphorylation assay using glutathione beads, ^{32}P-labeled GST-CTD, GST-RAP74c, and His_{10}-yFcp1. B: GST-RAP74c greatly enhances His_{10}-yFcp1 activity towards GST-CTD(^{32}P) in the matrix-based assay. GST-CTD(^{32}P) and GST or GST-RAP74c were coupled to glutathione beads. Free RAP74c was added for one experiment, and the reaction was initiated by adding 500 nM His_{10}-yFcp1. The reactions were incubated at room temperature and aliquots were removed at the indicated time points. The released ^{32}P was visualized by autoradiography. C: Quantification of the released ^{32}P. The signal of the released ^{32}P was quantified by analyzing the scanned films with the NIH image software. Arbitrary units (AU) were calculated by dividing the values for a given time point by the value for the 5 min time point of the GST control.

the phosphatase domain and the protein-interacting BRCT domain, are required to rescue Ess1/Ptf1 mutations in yeast. In order to verify the biological effect of Fcp1 for the Ess1/Ptf1 temperature-sensitive mutant yeast strain YPM2, that has been described by Hani et al. [14,39] and routinely used in our laboratory [7,12,18], we overexpressed the wild-type protein and different deletion mutants (Fig. 1A) at the permissive and the restrictive temperature. As shown in Fig. 1B, all proteins were expressed in YPM2 and migrated at the expected size in a SDS–PAGE gel.

YPM2 cells carrying plasmids for the expression of Fcp1 and its mutants did not show any growth effect under non-inducing conditions at the permissive temperature (Fig. 1C). However, changing the conditions to the restrictive temperature, only yeast cells expressing full length Fcp1 or Fcp1ΔNLS were able to survive. Therefore, deletion of the BRCT domain (Fcp1ΔBRCT) or deletion of the predicted phosphatase domain (Fcp1ΔPP) impairs the rescuing effect (Fig. 1C). Both domains have been described to be essential for Fcp1 [31]. The BRCT domain is required for the binding of Fcp1 to the transcription machinery via RAP74 [29,43] while the phosphatase domain is required for the catalytic action of Fcp1 [31]. Our results suggest that besides the phosphatase activity the proper localization of Fcp1 at the transcription machinery, mediated by the BRCT domain, is important to overcome the loss of Ess1/Ptf1.

3.2. Development of a novel in vitro assay to analyze the dephosphorylation of the CTD of the RNAP II by Fcp1

Today, a limited number of assays to analyze the dephosphorylation of the CTD are available. Mainly, these assays are based on the decreased mobility of the phosphorylated form of RNAP II in a SDS–PAGE or in the loss of radioactivity of ^{32}P-labeled RNAP II [27,28,44]. These assays require the purification of sufficient amounts of RNAP II from organisms like yeast, which makes the assays quite complex and difficult.

We developed an in vitro assay that uses only recombinant proteins and mimics the in vivo situation that occurs during the dephosphorylation of the CTD. One of the essential elements in the RNAP II complex is the interaction between RAP74 and the BRCT domain of Fcp1. This interaction localizes Fcp1 to the RNAP II complex bringing the phosphorylated CTD and Fcp1 in close proximity to each other. This close proximity of the CTD and Fcp1 seems to be a crucial factor for an efficient CTD dephosphorylation based on our observation that the BRCT domain of Fcp1 is required to suppress the Ess1/Ptf1 mutation in YPM2. Furthermore, Fcp1 gets activated by this interaction. As has been published recently [43,45], the very C-terminus of RAP74 is already sufficient for the interaction with and activation of Fcp1. In our assay we were able to achieve a similar situation by binding the different reaction partners to glutathione beads. After

coupling GST-CTD(^{32}P) and GST-RAP74(648–735) [43] to the beads, added Fcp1 binds to RAP74(648–735), which localizes Fcp1 close to CTD(^{32}P) and initiates the dephosphorylation (Fig. 2A).

Results of experiments using this assay are shown in Fig. 2B and quantified in Fig. 2C. Using only His_{10}-Fcp1 and GST as control, we detected only a weak ^{32}P signal similar to experiments where no glutathione beads were included in the assay (data not shown). When we added RAP74c to the dephosphorylation reaction, we detected a prominent increase in the ^{32}P signal (Fig. 2B,C), confirming an activation of FCP1 by RAP74c [43]. However, when we used GST-RAP74c the release of ^{32}P increased dramatically (Fig. 3B). Quantification of the ^{32}P signals reveals that, after 60 min incubation, the addition of free RAP74c or GST-RAP74c results in a 2- or more than 4-fold increase in the Fcp1 phosphatase activity, respectively (Fig. 2C). When we determined the initial rates of the Fcp1 reaction, the addition of free RAP74c results in a 4-fold increase (from 0.04 min^{-1} to 0.12 min^{-1}). Using GST-RAP74c in the assay, however, increased the initial rate more than 10 times when compared with the control reaction (from 0.03 min^{-1} to 0.37 min^{-1}). These results suggest that RAP74 had a dual effect, namely activation and targeting of Fcp1 to promote efficient dephosphorylation of the CTD. Furthermore, the strong activation of Fcp1 by GST-RAP74c indicates that our in vitro assay indeed seems to mimic the in vivo situation correctly.

3.3. *Pin1 accelerates the dephosphorylation of phosphorylated CTD by Fcp1 in vitro*

Based on the genetic interaction between Pin1 and Fcp1 we looked for a biochemical relationship between the two proteins. We investigated if the addition of Pin1 to the Fcp1 activity assay somehow affects the dephosphorylation of the CTD. Comparing experiments with and without Pin1 we observed a significant increase in the CTD dephosphorylation when Pin1 was present (Fig. 3A). Quantification of five independent experiments reveals that up to 20 min the control and the Pin1 reactions are nearly similar (Fig. 3B). However, after 30 min the signals become increasingly different, showing that Pin1 accelerates the CTD dephosphorylation by Fcp1 (Fig. 3B). It seems that Pin1 progressively enhances the dephosphorylation. The differences between the reactions with and without Pin1 become more pronounced at later time points, being highest at 60 min when we stopped the reaction. Here, Pin1 enhances the dephosphorylation about 2-fold (Fig. 3B). Although the effect of Pin1 on dephosphorylation of CTD by FCP1 is not dramatic, it is significant because it has previously been demonstrated that the effect of Pin1 on dephosphorylation by PP2A is about 2–3-fold [12]. Also, Pin1's effect on Cdc25c activity is about 1.2–1.5-fold [46].

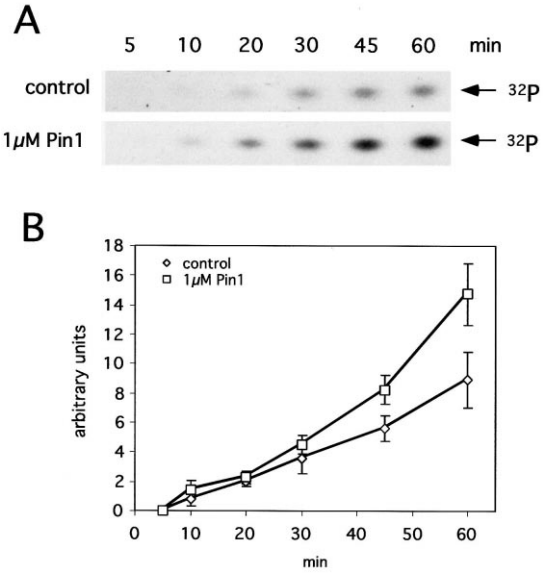

Fig. 3. Pin1 accelerates the dephosphorylation of GST-CTD(^{32}P). GST-CTD(^{32}P) and GST-RAP74c were coupled to glutathione beads. 1 μM Pin1 was added to the reaction and the dephosphorylation reactions were started with 500 nM His_{10}-yFcp1. The reactions were incubated at 10°C and aliquots were removed at the indicated time points. A: Autoradiograph and (B) quantification ($n = 5$) of dephosphorylation reactions with 1 μM Pin1.

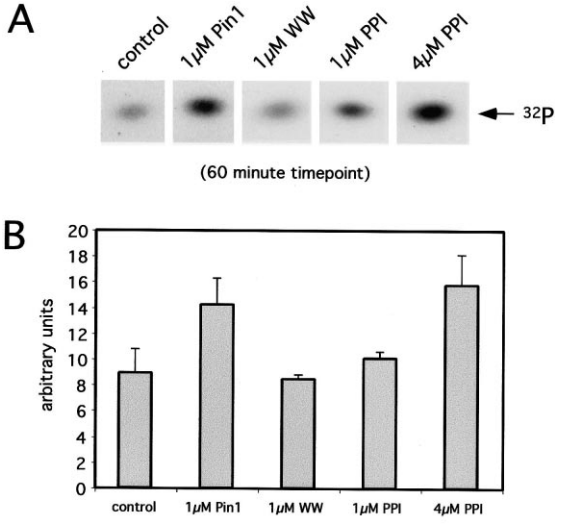

Fig. 4. The PPIase activity of Pin1 is sufficient to stimulate the dephosphorylation of GST-CTD(^{32}P). Pin1, the WW domain or the PPIase domain were added to the CTD dephosphorylation reaction described in Fig. 2. A: Autoradiograph and (B) quantification ($n = 5$) of the 60 min time point of the dephosphorylation reactions.

To examine whether the WW domain or the PPIase domain is required for Pin1 to affect CTD dephosphorylation, we added different truncation mutants of Pin1 to the reaction. As shown in Fig. 4, the addition of 1 μM WW domain did not increase the CTD dephosphorylation, whereas the PPIase domain alone was able to stimulate the reaction (Fig. 4). When we used a concentration of 1 μM PPIase domain, the effect was obvious but significantly lower than what we observed with 1 μM Pin1 (Fig. 4). However, after we increased the concentration of the PPIase domain to 4 μM, a signal similar to the reaction with 1 μM Pin1 was observed (Fig. 4). These results indicate that the PPIase activity of Pin1 is also sufficient to promote an efficient dephosphorylation of the CTD by Fcp1, provided the concentration of the PPIase domain in the assay is high. This is consistent with the previous report that the PPIase domain alone can accelerate the dephosphorylation by PP2A [12]. Therefore, the WW domain of the full length protein is probably required to target Pin1 to the phosphorylated CTD. The presented data are an additional example that the PPIase domain alone is required and sufficient to fulfill Pin1's function.

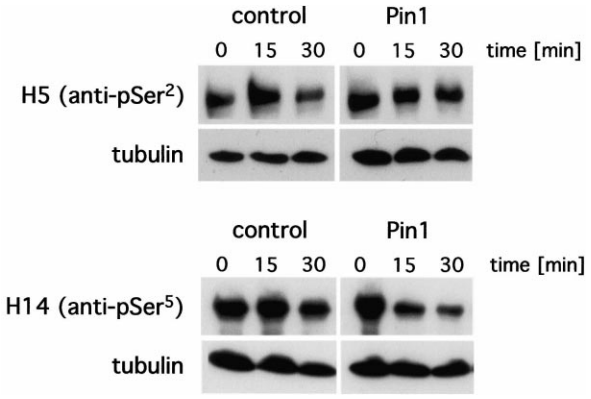

Fig. 5. Pin1 influences the phosphorylation of the CTD in vivo. YPM2 was transformed with pBC100 (control), Pin1/pBC100 (Pin1), Pin1-WW/pBC100 (WW) or Pin1-PPI/pBC100 (PPI) and grown at room temperature in glucose/galactose medium. The cultures were quickly brought to 37°C at an OD_{600} of about 0.8. Aliquots were removed at the indicated time points and the phosphorylation status of the CTD was analyzed by SDS–PAGE and Western blotting. A: Western blot for $pSer^2$ of the CTD with the specific antibody H5 and tubulin-loading control of the lysates. B: Western blot for $pSer^5$ of the CTD with the specific antibody H14 and tubulin-loading control of the lysates.

3.4. Pin1 influences the dephosphorylation of the CTD in vivo

In order to confirm the significance of Pin1 in the Fcp1 dephosphorylation reaction, we ectopically expressed Pin1 to replace yeast Ess1/Ptf1 to examine whether it somehow influences the phosphorylation status of the CTD in vivo.

We transformed YPM2 with plasmids that enable the galactose-induced expression of Pin1 [12]. Cultures growing in minimal medium with Glu/Gal were subjected to a 37°C heat shock to inactivate the endogenous heat-sensitive Ess1/Ptf1 [39]. Aliquots of the cultures were withdrawn at the indicated time points and analyzed for CTD phosphorylation by Western blotting (Fig. 5). We used the two antibodies H5 and H14, which specifically recognize either the $pSer^2$–Pro or the $pSer^5$–Pro epitope of the CTD, respectively [40]. Equal loading of the gels was confirmed by Coomassie staining of the blot (not shown) and by detecting yeast tubulin with the monoclonal antibody YOL1/34 (Fig. 5).

Our results clearly show that the expression of Pin1 was able to influence the phosphorylation state of the CTD. The effects, however, were different for Ser^2 and Ser^5 of the consensus sequence $Tyr^1Ser^2Pro^3Thr^4Ser^5Pro^6Ser^7$. As compared with the control experiment, the overall phosphorylation of Ser^2 was not changed significantly when Pin1 or its mutants were expressed, as detected by the H5 antibody (Fig. 5A). However, the analysis of the Ser^5 phosphorylation with the specific antibody H14 showed a different result (Fig. 5B). The phosphorylation status of the CTD on Ser^5 decreased significantly after 15 min growth at 37°C when Pin1 was expressed. The $pSer^5$ signal was even weaker after 30 min growth at the higher temperature. These results indicate that the Pin1 effect on the dephosphorylation of the CTD is different for Ser^2 and Ser^5 phosphorylation. While the phosphorylation of Ser^2 is not influenced, overexpression of Pin1 results in a decreased level of the $pSer^5$ epitope in vivo. This difference between $pSer^2$ and $pSer^5$ is consistent and can be explained with a recently published crystal structure of Pin1 and the phosphorylated form of the CTD heptapeptide [47]. The study shows that $pSer^5$ is required for the interaction with the WW domain of Pin1, whereas $pSer^2$ is not. It is known that the binding sites of Pin1 in Cdc25c and tau are also isomerized by Pin1 [12]. In analogy, this suggests that Pin1 preferentially targets the $pSer^5$–Pro bond of the CTD consensus and that Pin1 effects would be seen mainly at that position.

In summary, we have found that both the phosphatase domain and the BRCT domain of Fcp1 are required for its ability to rescue the Ess1 lethal phenotype. Based on this observation and other previous results, we developed a new in vitro assay to analyze the CTD dephosphorylation by Fcp1 that uses only recombinant proteins and mimics the in vivo situation of the CTD complex formation during the dephosphorylation of the CTD. This assay allows us to demonstrate for the first time that Pin1 accelerates dephosphorylation of the CTD by FCP1 in vitro and this effect is mediated by the PPIase domain. Finally, Pin1 also increases the dephosphorylation of the CTD preferentially on the Ser^5–Pro motif in yeast. These results demonstrate a new role for Pin1 in the regulation of CTD dephosphorylation.

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