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Research Article

Expression of the Stp1 LMW-PTP and inhibition of protein CK2 display a cooperative effect on immunophilin Fpr3 tyrosine phosphorylation and *Saccharomyces cerevisiae* growth

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Abstract. Although the yeast genome does not encode bona fide protein tyrosine kinases, tyrosine-phosphorylated proteins are numerous, suggesting that besides dual-specificity kinases, some Ser/Thr kinases are also committed to tyrosine phosphorylation in *Saccharomyces cerevisiae*. Here we show that blockage of the highly pleiotropic Ser/Thr kinase CK2 with a specific inhibitor synergizes with the overexpression of Stp1 low-molecular-weight protein tyrosine phosphatase (PTP) in inducing a severe growth-defective phenotype, consistent with a prominent role for CK2 in tyrosine phosphorylation in yeast. We also present in vivo evidence that immunophilin Fpr3, the only tyrosine-phosphorylated CK2 substrate recognized so far, interacts with and is dephosphorylated by Spt1. These data disclose a functional correlation between CK2 and LMW-PTPs, and suggest that reversible phosphorylation of Fpr3 plays a role in the regulation of growth rate and budding in *S. cerevisiae.*

Key words. Tyrosine-phosphorylated proteins; yeast; tyrosine kinase; immunophilin; CK2; LMW-PTP.

Tyrosine phosphorylation has been implicated in the regulation of crucial biological functions such as cell proliferation, differentiation, development, and transformation in higher eukaryotes [1]. The level of tyrosine phosphorylation is controlled by the concerted action of specific kinases and phosphatases. Phosphotyrosine protein phosphatases (PTPs) take part in such regulation either directly, via the dephosphorylation of phosphotyrosyl protein targets, or by down-regulating signal-transducing tyrosine kinases [2]. Although in *Saccharomyces cerevisiae* conventional tyrosine kinases are not present [3], tyrosine phosphorylation plays an essential role, e.g. in the regulation of mitogen-activated protein (MAP) kinase activity. These enzymes are activated by phosphorylation of both tyrosine and threonine residues by MEK (MAP kinase kinase), a dual-specificity protein kinase [4]. Anti-phosphotyrosine antibodies have also been shown to react with about 140 spots in a two-dimensional (2D) electrophoresis analysis of yeast lysates, suggesting that tyrosine phosphorylation in yeast may have more general relevance than just controlling the MAP kinase pathways [5]. Pertinent to this is also the observation that, although all protein kinases encoded by the yeast genome belong to the Ser/Thr family, 27 members of the family are able to phosphorylate poly-Glu-Tyr, a typical artificial substrate of *bona fide* tyrosine kinases [6]. On the other hand, a number of genes encoding putative PTPs have been reported in *S. cerevisiae*. Among PTPs, the members of the low-molecular-weight PTP subfamily (LMW-PTPs) are

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present not only in mammals but also in phylogenetically distant organisms, both prokaryote and eukaryote. Stp1 (small tyrosine phosphatase 1) is the *Schizosaccharomyces pombe* counterpart of the mammalian LMW-PTPs present ubiquitously in mammalian tissues [7]. Determination of the main kinetic constants of the recombinant Stp1 protein indicated that it has an enzymatic activity very similar to that of mammalian LMW-PTPs. LMW-PTPs possess the active-site motif (H/V)C(X)5R (S/T) and share the same catalytic mechanism of classical PTPs: the signature motif includes a cysteine and an arginine, involved in the formation of a phosphocysteinyl intermediate and in the stabilization of the transition state [8]. We have also obtained a Cys11 to Ser mutant of Stp1 that, similar to the C12S mammalian LMW-PTP mutant, has lost enzymatic activity but can still bind the substrate, thus behaving as a dominant negative protein [9]. Stp1 is 50% identical to its *S. cerevisiae* homologue, Ltp1, and its overexpression in *S. cerevisiae* induces an increase in the length of the unbudded phase if cells are grown in non-fermentable carbon sources, and an increase in heat shock resistance. By contrast, expression of the inactive C11S Stp1 mutant in *S. cerevisiae* promotes an increase in the number and intensity of Tyr-phosphorylated proteins in yeast lysates [5] consistent with its dominant negative potential with respect to endogenous Ltp1. This outcome supports the view that Stp1 and Ltp1 share the same protein targets and makes Stp1C11S a valuable tool for counteracting pTyr protein dephosphorylation in yeast and detecting endogenous substrates of Ltp1.

Among the Ser/Thr kinases that may play a role in tyrosine phosphorylation in yeast, CK2 (an acronym derived from the misnomer 'casein kinase 2') deserves special attention due to its pleiotropy and its unambiguous implication in Tyr phosphorylation of at least one yeast protein, immunophilin Fpr3 [10]. CK2 is a ubiquitous, constitutively active kinase which phosphorylates more than 300 proteins implicated in a variety of cellular functions, with special reference to signal transduction, gene expression, protein synthesis, and metabolic regulation [11]. Among all these proteins, *S. cerevisiae* immunophilin Fpr3 is exceptionally phosphorylated by CK2 at tyrosine184 [12]. Peptide substrates reproducing the sequence surrounding Fpr3 Tyr184 were also phosphorylated in vitro by CK2, revealing a consensus sequence similar but not identical to that of its Ser/Thr substrates [13]. Such an ancillary tyrosine kinase activity of CK2 may play a relevant role in yeast, due to the fact that bona fide tyrosine kinases are absent, and Ser/Thr kinases are therefore expected to catalyze Tyr phosphorylation of proteins. In turn, these Tyr-phosphorylated proteins are substrates for a number of bona fide tyrosine phosphatases encoded by the yeast genome. Although to date the only known tyrosine-phosphorylated CK2 substrate is immunophilin Fpr3, several tyrosine-phosphorylated proteins, which are substrates of the LMW-PTPase, have been shown to contain potential tyrosyl phosphoacceptor sites conforming to the consensus sequence recognized by CK2 [5]. *S. cerevisiae* appears therefore to be the first-choice organism for a systematic search of Tyr-phosphorylated CK2 protein targets. Although in yeast the deletion of both catalytic CK2 subunits is lethal [14], CK2 activity can be selectively attenuated using a highly specific inhibitor, tetrabromobenzotriazole (TBB), which has no effect on a variety of other protein kinases [15].

Beside being an atypical substrate of CK2, Fpr3 is also the only tyrosine-phosphorylated protein so far identified in yeast that is not itself a protein kinase. Fpr3 belongs to the FK506-binding protein family (FKBPs) endowed with prolyl isomerase catalytic activity [16]. Peptidylprolyl-cis, trans-isomerases (PPIases) are thought to participate in protein folding and protein assembly events in vivo, because in vitro these enzymes catalyze interconversion of cis and trans isomers of the bond involving the imino nitrogen of proline in peptide and protein substrates [17]. These enzymes are highly conserved from bacteria to yeast and humans. They are found in multiple intracellular compartments and have been suggested to play a critical general role in protein folding. Even if their presence is not required for growth, they may perform specific functions through interaction with partner proteins [18]. Fpr3 was originally identified as a nuclear-localization-sequence-binding protein, a nuclear envelope protein, and as an FK520-binding protein. Interestingly, Fpr3 is localized to the nucleolus, where CK2 also colocalizes with several of its physiological targets [10]. These observations prompted us to start an investigation aimed at disclosing a possible functional correlation between LMW-PTP and CK2, which would corroborate the concept that CK2 plays an important role as a tyrosine-phosphorylating agent in yeast. We decided to start by focusing on the only Tyr-phosphorylated CK2 substrate identified to date, by checking if immunophilin Fpr3 might also be a substrate of LMW-PTPs. Here we show that immunophilin Fpr3 indeed interacts with Stp1 and exhibits a negligible level of tyrosine phosphorylation in Stp1-expressing yeast cells when treated with a CK2 specific inhibitor. We also provide evidence that such a drastic reduction in Fpr3 tyrosine phosphorylation, promoted by the concerted action of CK2 inhibition and Stp1 overexpression, is accompanied by defective growth, consistent with the concept that pTyr dephosphorylation of Fpr3 and/or of other protein substrates common to CK2 and LMW-PTPs is detrimental to yeast growth.

Materials and methods

Strains, growth media, and plasmids

The yeast strains used in this study are described in table 1.

Table 1. Yeast strains used.

Strain	Genotype	Source and reference
w303-1A	MAT**a** leu2-3,112ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100, GAL SUC mal	[25]
w303pAAH5	w303-1A + pAAH5	[5]
w303pAStp1	w303-1A + pAAH5-Stp1	[5]
w303pAStp1C11S	w303-1A + pAAH5-Stp1C11S	[5]
w303pyFpr3	w303-lA + yEP351-Fpr3	this study
JK93da	MAT**a** his4 HMLa leu2-3, 112 rme1 trp1 ura3-52	[26]
JK93da + pAAH5	JK93dapAAH5	this study
JK93dapAStp1	JK93da + pAAH5-Stp1	this study
KDY86.6a	Δfpr3::URA3(JK93da)	[18]
KDY86.6apAAH5	KDY86.6a + pAAH5	this study
KDY86.6apAStp1	KDY86.6a + pAAH5-Stp1	this study
BY4743	MAT**a** his3-1, leu2, lys2, ura3-52 Δltp1::KANmx	M. Tommasino

The parent strain is w303-1A MATa leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal. Plasmids pAAH5 containing Stp1 or its dominant negative Stp1C11S cDNA were constructed as previously described [5] and used for transformation of w303-1A to obtain strains w303pAStp1 and w303pAStp1C11S, respectively. The plasmid yEP351-Fpr3, used to obtain the overexpressing Fpr3 strain, was a gift from J. Thorner [16].

Strain KDY86.6a lacking the Fpr3 gene and its isogenic wild-type strain JK93da were gifts from J. Heitman [18]. The Ltp1 deletion strain was a kind gift from M. Tommasino.

Cells were grown at 30 °C either in YP medium [1% yeast extract and 2% peptone (Biolife, Italy)] or in synthetic minimal medium (SD) containing 0.67% w/v YNB (Difco, Life Technologies, USA) and appropriate quantities of the 'drop-out' amino acid-nucleotide mixture (Bio101). 2% w/v glucose was added as carbon source. Solid medium contained 2% w/v agar.

Growth of liquid cultures was monitored as an increase in optical density (OD) at 600 nm. The fraction of budded cells was scored by direct microscopic observation on at least 300 mildly sonicated cells. The formula used to calculate T_B (the length of the budded phase) was $T_B = \log2(1 + F_B)T$, where F_B is the percentage of the budded cells and T is the population doubling time ($T = \ln2/\alpha$, where α is the experimentally determined growth rate).

Immunoprecipitations and immunoblotting

Protein lysates were prepared in 500 µl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonylfluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin). Cells were broken with glass beads in a Fastprep instrument (Savant, QBiogene Inc., USA) and protein extracts were clarified by centrifugation at 13,000 rpm for 2 min. The lysates were immunoprecipitated for 4 h at 4 °C with 0.1 µg of the specific antibody. Immunocomplexes were collected on protein A-Sepharose (Amersham Pharmacia Biotech, UK), separated by gel electrophoresis (SDS-PAGE) and transferred by electroblotting to polyvinylidene difluoride membrane (PVDF) (Immobilon P; Millipore, Bedford, Mass.). Immunoblot analysis was performed using either anti-PY20 antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif.) at a 1:1000 dilution, rabbit polyclonal anti-Stp1 antibody at 1:1000 dilution or rabbit polyclonal anti-Fpr3 antibody at 1:5000 dilution. The anti-Fpr3 antibodies were a gift from Prof. J. Thorner [16]. Antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies. Immunoblots were visualized using ECL reagents (Amersham Pharmacia Biotech). Western blot analyses were quantitated by densitometric analysis. This analysis was performed with the Bio-Rad Gel Doc 2000 Chemi Doc, using Quantity One quantitation software.

Treatment with TBB

SD medium was supplemented with TBB prepared as described by Zien P. et al. [19] at a final concentration of 10 µM in DMSO. Cells were grown until the stationary phase was reached and aliquots of cells were collected at the indicated time.

Results

Fpr3 coimmunoprecipitates with and is a substrate of Stp1

Stp1, a *S. pombe* LMW-PTP, is 50% identical to its *S. cerevisiae* homologue Ltp1. Its catalytically inactive mutant Stp1C11S has been shown to act as an effective dominant negative with respect to Ltp1. Therefore, to acquire information about the possible role of LMW-PTP in Fpr3 dephosphorylation, we first wanted to check whether Stp1 and Fpr3 interact in vivo with each other, using a coimmunoprecipitation assay. Cell lysates from w303 cells overexpressing Stp1 and from an empty vec-

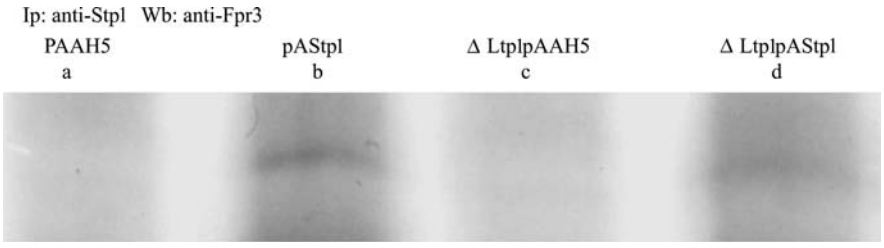

Figure 1. Fpr3 coimmunoprecipitates with Stp1. Lysates prepared from w303pAAH5 (empty vector) (a), w303pStp1 (b), w303ΔLtp1pAAH5 (c), and w303ΔLtp1pAStp1 (d) strains were subjected to immunoprecipitation with anti-Stp1 antibodies and the immunoprecipitated proteins were examined by immunoblotting with anti-Fpr3 antibodies.

tor control strain (not expressing Stp1) were separately subjected to immunoprecipitation with anti-Stp1 antibodies and immunoprecipitates were resolved by SDS-PAGE. Immunoblots using anti-Fpr3 antibodies revealed the presence of the Fpr3 protein only in the immunoprecipitates of the strain expressing Stp1 (fig. 1, lane b). To rule out the possibility that the interaction of Fpr3 with Stp1 might be affected by endogenous *S. cerevisiae* Ltp1, we performed the same experiment in a yeast mutant strain lacking the *S. cerevisiae* homologue of Stp1 [deleted for Ltp1 (ΔLtp1)] and in which Stp1 is overexpressed. As shown in figure 1 lane d, a sharp band corresponding to the Fpr3 immunoprecipitated protein is also present under these conditions. Therefore, Fpr3 interacts in vivo with Stp1. To verify that Fpr3 not only interacts with but is also a substrate of LMW-PTPs, lysates from empty vector controls (w303pAAH5) and from strains expressing either active Stp1 (w303pStp1) or its catalytically inactive protein Stp1C11S (w303pStp1C11S) were immunoprecipitated with anti-Fpr3 antibodies. An anti-phosphotyrosine immunoblot was then performed (fig. 2A). The blot was stripped and reprobed with anti-Fpr3 antibodies to quantitate the coimmunoprecipitation of Fpr3 with Stp1 and its dominant negative molecule Stp1C11S (fig. 2B). The amount of phosphorylated Fpr3 was evaluated by normalizing the data from immunoblots A and B (fig. 2) using Chemidoc Quantity One softwear. The results shown in figure 2C demonstrate that overexpression of active Stp1 (lane b) results in a drop in tyrosine phosphorylation of Fpr3, whereas the expression of catalytically inactive Stp1C11S (lane c) leads to a significant increase in Fpr3 tyrosine phosphorylation. Additional evidence that Stp1 is indeed responsible for dephosphorylation of Fpr3 was provided by anti-phosphotyrosine immunoprecipitation followed by an anti-Fpr3 immunoblot. As shown in figure 2D, a lower amount of Tyr-phosphorylated Fpr3 is present in the strain overexpressing Stp1. In contrast, pTyr-Fpr3 is remarkably increased in the strain expressing the catalytically inactive Stp1. Collectively, these data show that Tyr-phosphorylated Fpr3 is an in vivo substrate of Stp1. In fact, Fpr3 phosphorylation is decreased by Stp1 overexpression on the one hand and increased by expression of its catalytically inactive mutant C11S on the other.

Stp1 overexpression and CK2 inhibition influence cell growth and cell cycle parameters

To determine if simultaneous CK2 inhibition and Stp1 overexpression might affect yeast growth parameters in a synergistic way, we first determined cell growth kinetics of a control yeast strain harboring the empty vector or a yeast transformed with an Stp1 overexpression vector in the presence of the highly selective, cell-permeable CK2 inhibitor TBB [15].

For this purpose, these cells were grown for 24 h in liquid medium in the presence of 10 μM TBB. Analyses of their growth kinetics (fig. 3) show that growth is slightly affected by Stp1 overexpression alone, but this effect is dramatically enhanced by concomitant addition of the specific CK2 inhibitor TBB (empty circles in figure 3), which does not have an inhibitory effect on the growth of the control strain. Upon addition of 10 μM TBB to Stp1-expressing cells, the lag phase becomes much longer. This longer lag phase, observed for 8 h after TBB treatment, could correlate with a reduced level of Fpr3 phosphorylation. In figure 3, the duplication time and the percentage of budded cells in Stp1-expressing cells, either treated or not with TBB, are shown. These results show that when cells are grown in the presence of TBB, the number of budded cells is increased in Stp1-overexpressing cells. As shown in table 2, the duplication time increased, affecting both the S, G2 and M phase (budded phase) and the G1 phase (unbudded phase) of the cell cycle, as indicated by the ratio between the length of the budded phase and the duplication time. This ratio has similar values (about 0.5) in all the strains either treated or not with TBB. In the Stp1-expressing strain, however, bud emergence could be observed 90 min earlier, compared to the isogenic control, whereas if TBB was also added, the duplication time was dramatically increased with a delay of about 5 h. To assess the possible role of Fpr3 phosphorylation on growth rate, we analyzed the effects of Stp1 overexpression and TBB-dependent CK2 inhibition on the Fpr3 null mutant strain KDY86.6a in com-

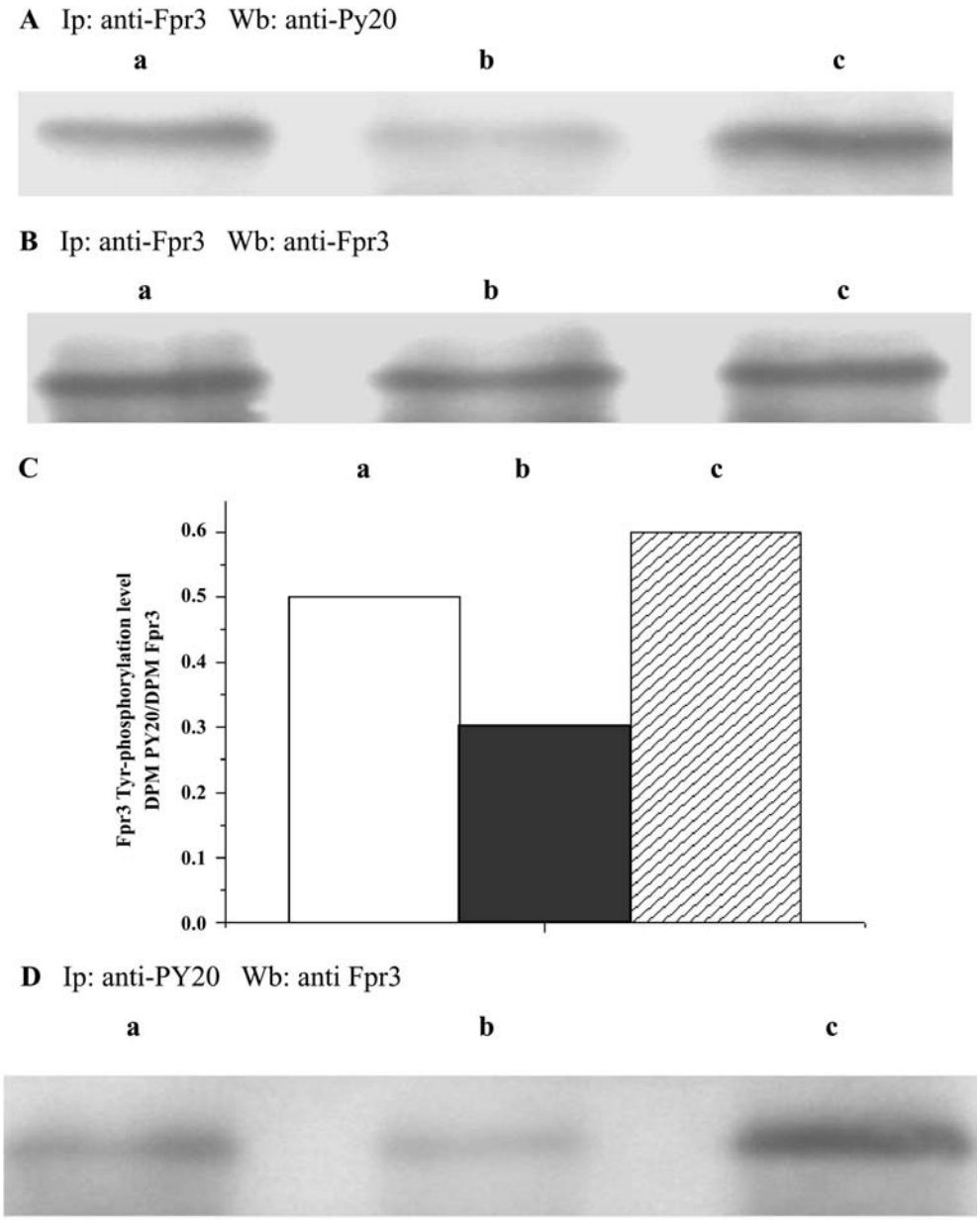

Figure 2. Stp1 controls Fpr3 tyrosine phosphorylation level in vivo. Lysates from w303pAAH5 (empty vector) (a), w303pStp1 (b), and w303pStp1C11S (c) strains were subjected to immunoprecipitation with anti-Fpr3 antibodies and analyzed by immunoblot with anti-phosphotyrosine antibodies (*A*). The blot was stripped and reprobed with anti-Fpr3 antibodies (*B*). Quantitative evaluation of the Fpr3 phosphorylation level was normalized by densitometric analysis (*C*) using Quantity One quantitation software. The same lysates were immunoprecipitated with anti-phopshotyrosine antibodies and the immunoprecipitates were analyzed by immunoblot with anti-Fpr3 antibodies (*D*).

parison to the wild-type strain (JK93da). All the strains (JK93dapAAH5 empty vector control strain, JK93dapAStp1 control strain expressing Stp1, KDY86.6apAAH5 empty vector control strain Fpr3 deletion, KDY86.6apAStp1 Fpr3 deletion expressing Stp1) were grown in liquid media. Their growth rates were examined upon TBB treatment in comparison to untreated controls. Under these conditions, the null Fpr3 mutant showed no differences in duplication time (table 2), consistent with the view that even though the immunophilins are not essential for yeast, the dephosphorylated form of Fpr3 could be the cause of the reduced growth rate. To note is that no significant differences in duplication times can be observed (data not shown) when cell growth kinetics of w303pYFpr3 cells, overexpressing Fpr3 in the presence of TBB, were compared to that of the empty vector control strain.

Table 2. Effect of Stp1 overexpression on growth and cell cycle parameters of strain w303 exponentially growing on YDP medium treated or not with 10 μM TBB.

Yeast strain	T	TB	TB/T	T (+TBB)	TB (+TBB)	TB/T (+TBB)
w303 pAAH5	165 ± 10	87 ± 6	0.53	176 ± 5	89 ± 5	0.50
w303pStp1	183 ± 15	100 ± 12	0.54	494 ± 7	240 ± 19	0.48
w303yEP351-FPR3	150 ± 16	81 ± 9	0.54	177 ± 6	97 ± 17	0.54
JK93dapAAH5	100 ± 10	58 ± 15	0.58	120 ± 10	65 ± 15	0.54
JK93dapAStp1	99 ± 4	55 ± 12	0.55	350 ± 15	180 ± 15	0.51
KDY86.6apAAH5	197 ± 12	107 ± 7	0.54	205 ± 12	108 ± 12	0.52
KDY86.6apAStp1	197 ± 15	108 ± 16	0.54	215 ± 2	110 ± 21	0.51

The strains used are: JK93dapAAH5, empty vector control strain; JK93dapAStp1, control strain overexpressing Stp1; KDY86.6apAAH5, empty vector control strain Fpr3 deletion; KDY86.6apAStp1, Fpr3 deletion overexpressing Stp1. Data are average ± SD from four independent experiments. The duplication time (T) and length of the budded phase (TB) are expressed in minutes.

Stp1 overexpression and CK2 inhibition affect Fpr3 tyrosine phosphorylation

The above data indicate that overexpression of Stp1 PTP and concomitant inhibition of CK2 leads to a reduced growth rate. Moreover, the observation that under these conditions Fpr3 Tyr phosphorylation drops to negligible values points toward the involvement of the Fpr3 phosphorylation state in the control of yeast growth, especially considering that an Fpr3 null mutant strain has lost responsiveness to the synergistic effect of Stp1 and TBB on growth. As shown in figure 2, Stp1 overexpression alone leads to a 40% reduction of Fpr3 Tyr phosphorylation. The residual Fpr3 phosphorylation could be due to concomitant rephosphorylation by protein kinase(s). Since Fpr3 phosphorylation has been reported to be mediated by protein kinase CK2 [12], we reasoned that blockage of CK2 activity by the selective inhibitor TBB could cooperate with Stp1 overexpression to further reduce Fpr3 Tyr phosphorylation. To check this possibility, we determined the Tyr phosphorylation level of Fpr3 either in the presence or absence of TBB in empty vector control yeast and in yeast transformed with Stp1, using a coimmunoprecipitation assay. Cell lysates from w303 cells overexpressing Stp1, and from its empty vector control strain, either treated (fig. 4, lane a, b) or not treated (fig. 4, lane c, d) with TBB, were subjected to immunoprecipitation with anti-Fpr3 antibodies and an anti-phosphotyrosine Western blot was then performed (fig. 4A). Equal amounts of Fpr3 were immunoprecipitated (fig. 4B). Indeed, the results show that Stp1 overexpression, in

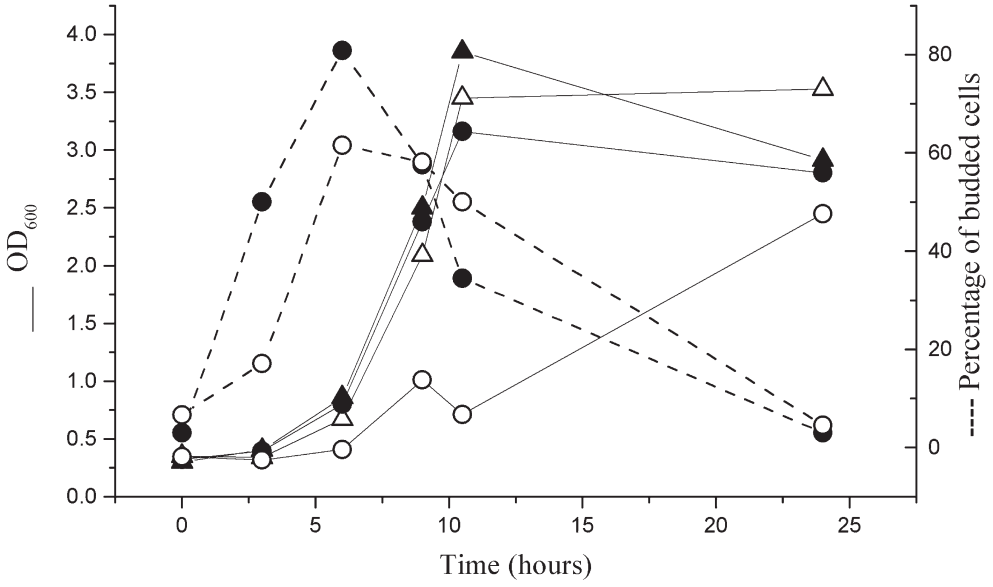

Figure 3. Stp1 overexpression and CK2 inhibiton influence cell growth kinetics and cell cycle parameters. w303pAAH5 (empty vector) (triangles) and w303pStp1 (circles) strains were grown at 30 °C in minimal medium for 24 h. At time point 0, cells were treated with 10 μM TBB (empty symbols) or not treated (closed symbols). At the indicated times, samples were taken for determination of OD_{600} (solid line) and the percentage of budded cells (dashed line) as described in Materials and methods. The experiment was repeated three times with similar results. The results of a single typical experiment are reported. The percentage of budded cells is reported only for the w303pStp1 strain. For complete data see table 1.

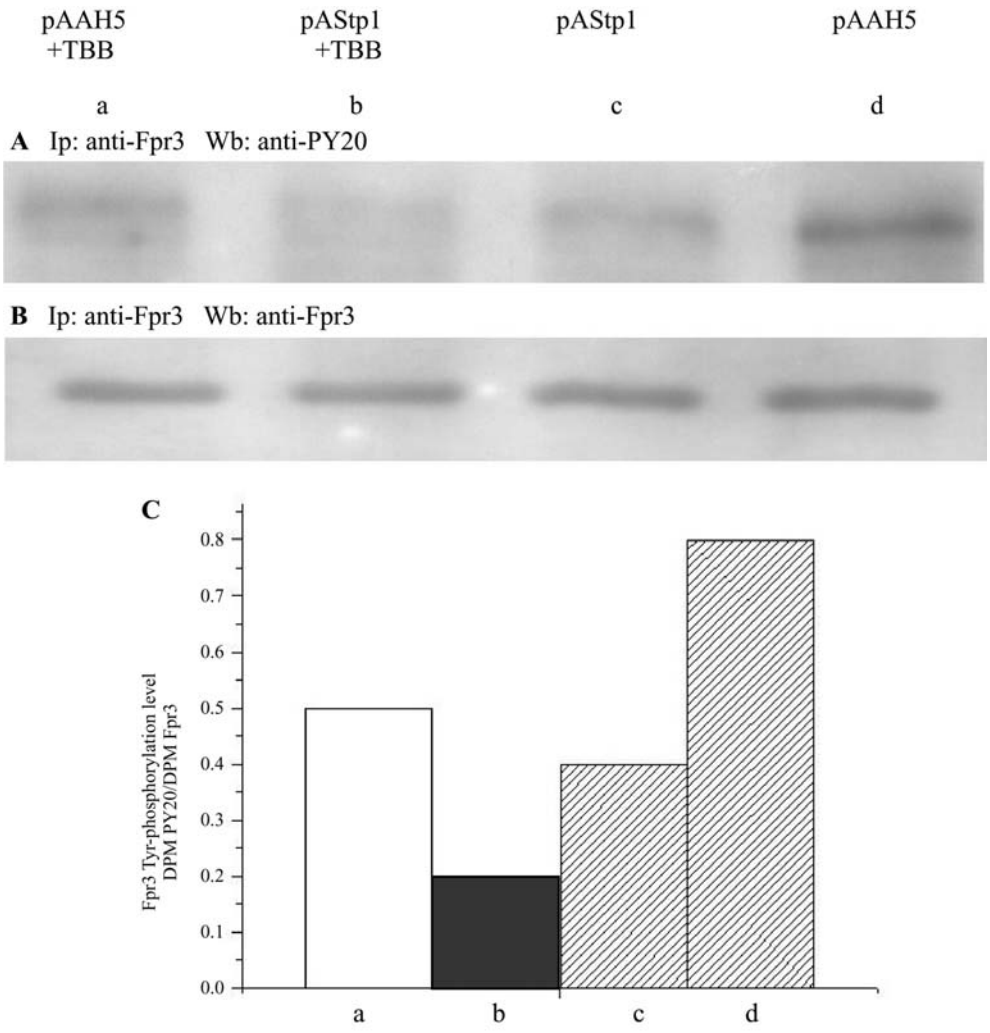

Figure 4. Stp1 overexpression and CK2 inhibition control Fpr3 tyrosine phosphorylation level in vivo. TBB 10 µM was added for 8 h to growing w303pStp1 (a) and w303 pAAH5 (b) strains; (c) and (d) represent the control strains without TBB. Lysates were subjected to immunoprecipitation with anti-Fpr3 antibodies and the immunoprecipitates were analyzed with anti-phosphotyrosine antibodies (*A*). The blot was stripped and reprobed with anti-Fpr3 antibodies (*B*). The band corresponding to Fpr3 is shown. Data were analyzed by Chemidoc Quantity One software and the normalized data are shown (*C*).

conjunction with TBB treatment for 8 h, leads to a dramatic drop in Fpr3 phosphotyrosine content (fig. 4C compare bars b and d). We performed the experiment after 8 h of TBB treatment because the longest lag phase is observed at this time, as described above. In contrast, incubation with TBB (lane a) or overexpression of Stp1 (lane c) alone decreased the level of Tyr-phosphorylated Fpr3 to a lesser extent (fig. 4C, bars a and c, respectively). The residual Tyr phosphorylation of Fpr3 observed in the strain transformed with Stp1 and treated with TBB (about 25% of control) may be due to incomplete inhibition of endogenous CK2 by 10 µM TBB. In fact, in Jurkat cells, the TBB concentration had to be raised up to 75 µM to induce an almost complete inhibition of endogenous CK2 [20]. Alternatively, dual-specificity Ser/Thr kinases other than CK2 might contribute to Fpr3 Tyr phosphorylation. In any case, our results indicate that both Stp1 overexpression and CK2 inhibition are involved in reducing Fpr3 Tyr phosphorylation to a minimal level.

Discussion

Previous experiments indicated that overexpression of the *S. pombe* LMW-PTP Stp1 in *S. cerevisiae* deeply affects the yeast phosphoproteome, as monitored by 2D electrophoretic protein separation [5]. Stp1 is a shares 50% identity with its *S. cerevisiae* homologue, Ltp1, which suggests that they may display similar biological effects by targeting homologous phosphoproteins in different organisms. In a previous study, we successfully used Stp1 and its catalytically inactive C11S mutant as a tool to study the tyrosine phosphorylation level of the *S. cere-*

visiae proteome. Immunoblotting analysis of the tyrosine-phosphorylated protein profiles in the different recombinant strains allowed us to identify putative Ltp1 endogenous substrates and prompted us to investigate in closer detail the physiological effects of Stp1 overexpression.

An added value of this investigation would be to shed light on the mechanism by which the Stp1/Ltp1 targets are phosphorylated in yeast. The yeast genome does not in fact encode any bona fide protein tyrosine kinase and, therefore, the remarkable number of Tyr-phosphorylated proteins detectable in this organism [5] must be generated through the intervention not only of canonical dual-specificity kinases (essentially MEKs, whose targeting is restricted to MAP kinases) but also Ser/Thr kinases, which in a number of cases have been shown able to phosphorylate tyrosyl residues as well, albeit with reduced efficiency [20]. Pertinent to this, a recent systematic analysis of *S. cerevisiae* kinase specificity revealed that 27 Ser/Thr kinases are able to phosphorylate poly(Glu, Tyr) 4:1, a broad-specificity artificial substrate of tyrosine kinases [6].

While for most of these kinases, their actual physiological implication in tyrosine phosphorylation remains a matter of conjecture, in at least one case, this implication has been unambiguously proven. Yeast immunophilin Fpr3 is in fact phosphorylated in vitro and in vivo at both Ser and Tyr residues by CK2, a very pleiotropic Ser/Thr kinase whose continuously increasing list of targets includes more than 300 proteins implicated in a wide variety of cellular functions [10, 11]. More than 300 residues affected by CK2 in its substrates have been identified, all specified by multiple acidic side chains, with those at position n + 3 and n + 1 playing the most important role. The great majority of these phosphorylated residues are serines, 40 or so are threonines and one, Tyr184 of immunophilin Fpr3, is a tyrosine. A priority in our study was therefore to assess whether Tyr phosphorylation of Fpr3, catalyzed by CK2, is reversed by LMW-PTPs. This appears to be the case, since Fpr3 both interacts with and is dephosphorylated by Spt1, under in vivo conditions (see figs 1, 2).

As far as the functional consequences of Fpr3 dephosphorylation are concerned, particularly remarkable is the observation that under conditions that maximally reduce tyrosine phosphorylation (simultaneous inhibition of CK2 and overexpression of Stp1), the duplication time of yeast cells is increased threefold. Whether and to what extent this delay is actually mediated by Fpr3 remains an open question. Elucidation of this point will require on the one hand the identification of other yeast proteins whose phosphorylation might be affected in a concerted way by CK2 and LMW-PTPs, and on the other, a better understanding of the biochemical functions of Fpr3 and, more generally, of proteins belonging to the FKBP family endowed with prolyl isomerase activity. All these topics are still poorly understood and it is therefore premature to speculate about the regulation of Fpr3 biological activities by tyrosine phosphorylation. Our data are anyway consistent with the view that Fpr3 Tyr phosphorylation level may affect yeast growth rate.

By disrupting each of the 12 individual immunophilin genes, Dolinski K. et al. [18] showed that none of them are essential for viability. Nevertheless, an important role of Fpr3 Tyr phosphorylation in cell growth is highlighted by our experiments with the Fpr3 null mutant: this mutant proved in fact to be insensitive to the cooperative effect of Stp1 overexpression and TBB treatment which conversely have detrimental consequences on the growth of wild-type yeast. This observation strongly suggests that the presence of unphosphorylated (but not of phosphorylated) Fpr3 is harmful to growth rate and budding. A conceivable scenario would be that an increased level of unphosphorylated Fpr3 might lead to the accumulation of misfolded proteins which in turn could act as a signal inducing a stress response and growth arrest. In contrast, the absence of Fpr3 protein would not be detrimental, due to the redundancy of other immunophilins. In other words, Fpr3 would behave as a dominant negative unless it is converted into its phosphorylated form.

Our observations corroborate the view that CK2 operates in yeast as a tyrosine kinase [21]. This also means that it could be committed to Tyr phosphorylation of numerous proteins beside immunophilin Fpr3. While our data establish a clear link, in yeast, between CK2 and LMW-PTPs, responsible for the phosphorylation and dephosphorylation of Fpr3, respectively, their antagonistic effect on yeast growth may be mediated by a more complex network of reversible protein Tyr phosphorylation. In this respect, the Tyr phosphorylation state of Fpr3 could represent a useful indicator of how CK2 and LMW-PTPs cooperate in a concerted manner to regulate the level of tyrosine phosphorylation in yeast, without providing, however, a comprehensive view of the signaling pathways which are under the combined control of CK2 and LMW-PTPs.

These new data add a new level of complexity to the striking pleiotropy of CK2, disclosing the possibility that beside its endless list of Ser/Thr protein targets, a new list of Tyr-phosphorylated proteins is awaiting compilation. Moreover, they may provide a clue to ways to control the effects, though not the activity, of CK2. An intriguing feature of this kinase is in fact its high constitutive activity, independent of any of the many devices that control the activity of other kinases, except in response to specific stimuli [22]. There have been proposals [23] that the control lacking to CK2 might be exerted by the phosphatases acting on its phosphorylated substrates. In this respect, LMW-PTPs, susceptible to such a multifarious regulation through phosphorylation, second messengers and redox

state [24], would represent suitable partners to cope with the constitutive activity of CK2.

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