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The in vivo tyrosine phosphorylation level of yeast immunophilin Fpr3 is influenced by the LMW-PTP Ltp1

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Abstract

Tyr-phosphorylation in *Saccharomyces cerevisiae* is essential in controlling the activity of MAP kinase regulating mating, pseudohyphal growth, and cell wall biosynthesis. Yeast serves as a model system for studying the biological function of many protein kinases and PTPs. Two LMW-PTP from yeast have been cloned, namely, Ltp1 from *S. cerevisiae* and Stp1 from *Schizosaccharomyces pombe*. The sequences of both enzymes are relatively similar to those of the mammalian LMW-PTP. Recently we showed that the yeast immunophilin Fpr3 interacts with Stp1 and its dephosphorylated state induces a growth defective phenotype. Here we show the phosphatase activity of Ltp1 on Fpr3 and we demonstrated that Tyr 184 is the residue phosphorylated on in vivo Fpr3. We also described the marked activation of Ltp1 by adenine in *S. cerevisiae* proteome and determined in vivo the influence of tyrosine phosphorylation on Fpr3 localization.

Keywords: LMW-PTP; *Saccharomyces cerevisiae* Ltp1; Immunophilin Fpr3; Tyrosine phosphorylation in yeast

Phosphotyrosyl protein phosphatases are structurally diverse enzymes that have fundamental roles in cellular processes, including effects on metabolism, cell proliferation, and differentiation [1]. Although in budding yeast conventional tyrosine kinases are not present, Zhu et al. [2] using a chip technology identified 27 kinases able to phosphorylate poly-Glu-Tyr, a typical artificial substrate of bona fide tyrosine kinases. Moreover, Tyr-phosphorylation has been shown in *Saccharomyces cerevisiae* to be essential in controlling the activity of MAP (mitogen-activated-protein) kinases which, in turns regulate mating, osmosensing pseudohyphal and invasive growth, spore wall assembly, and cell wall biosynthesis [3]. The low-molecular weight protein tyrosine phosphatases (LMW-PTP) are single-domain cytosolic enzymes with molecular masses of approximately 18 kDa. The active site sequence motif of these enzymes has a conserved (V/I)CXGNXCRS sequence. Members of this family have been identified in a wide variety of organisms, including bacteria, yeast, rat, bovine, and human [4–8], suggesting that these enzymes have important cellular functions. Yeast serves as a simple, but often useful, model system for studying the biological function of many protein kinases and PTPs. Two LMW-PTP from yeast have been cloned, namely, Ltp1 from *S. cerevisiae* [9] and Stp1 from *Schizosaccharomyces pombe* [10]. The sequences of both enzymes are relatively similar to those of the mammalian LMW-PTP, with identity of 42% and 39% for the Stp1 and Ltp1, respectively. The physiological role of the yeast enzymes is still unknown but the mammalian LMW-PTP shows a broad range of

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substrates including the PDGF and insulin receptors [11], caveolin [12], and the p190Rho GAP [13]. The activation of mammalian LMW-PTP by purines or purine derivatives, nucleosides, and nucleotides has been described [14–16], together with the activity modulation of human LMW-PTP by adenine and hypoxanthine [17]. The mechanism of the activation of the LMW-PTP by purines is not completely understood. In a recent paper studying the structural basis for the activation of the Ltp1, Wang et al. [18] suggested that adenine molecule activates the enzyme by binding in the active site during the dephosphorylation step, showing the existence of a specific site on the enzyme that is capable to bind the purine effectors. In order to identify a possible role of the LMW-PTP in yeast we demonstrated that the expression of the Stp1 LMW-PTP, or of its dominant negative mutant, in *S. cerevisiae*, affects the phosphoproteome of this organism [19]. Moreover, in specific stress conditions, yeast strains that express Stp1 show a phenotype correlated with low cAMP level, suggesting an involvement of the protein phosphorylation in the regulation of cAMP pathway (unpublished data). All these data indicate that tyrosine phosphorylation in budding yeast is a more relevant process than previously suspected.

In a recent paper we showed that the yeast immunophilin Fpr3 interacts with Stp1 and exhibits a negligible level of tyrosine phosphorylation in Stp1 expressing yeast cells if treated with a CK2 specific inhibitor [20]. A drastic reduction in Fpr3 tyrosine phosphorylation, promoted by the concerted action of CK2 inhibition and Stp1 expression, is accompanied by defective growth. Fpr3 is a peptidylprolyl-*cis*,*trans*-isomerase (PPIase) acting in conjunction with protein chaperones, accelerating the rate of conformational interconversions around proline residues in polypeptides [21]. The functions of PPIase are still poorly understood, even if many of them assist in the folding (or unfolding) of individual proteins. Folding of many cellular proteins is facilitated by molecular chaperones. Analysis of both prokaryotic and lower eukaryotic model systems has revealed the presence of ribosome-associated molecular chaperones, thought to be the first line of defense against protein aggregation as translating polypeptides emerge from the ribosome [22]. In *Drosophila melanogaster* a mutation in a cyclophilin causes misfolded forms of rhodopsin [23]. PPIase are also required for the assembly of protein–protein complexes. Even if *fpr3* null mutations had no discernible phenotype [24], cells might be more sensitive to stress conditions accumulating higher level of misfolded proteins and aggregated unassembled proteins than wild-type cells, which might act as a signal to induce stress response. Localization of Fpr3 in the nucleolus suggests possible roles of this PPIase in the folding of ribosomal proteins, in the assembly of pre-ribosomes or in the export of ribosomes to the cytoplasm [25]. Furthermore, like other nucleolar proteins, it is phosphorylated in vivo. This modification may have a consequence for the activity or localization of Fpr3. The LMW-PTP from the yeast *S. cerevisiae*, Ltp1, shows substrate specificity toward artificial substrates that is generally similar to that exhibited by the homologous mammalian enzymes [9]. However, it is even more activated by adenine, bringing its activity to a level comparable with those of its vertebrate counterparts [18]. Recently, two residues (W135 and Y136) were replaced by site-directed mutagenesis to assess the role of some residues, in the active site, in substrate recognition. Paoli et al. described the kinetic properties of these mutants and demonstrate that the molecular site, which determines adenine dependent activation, is also involved in substrate recognition (unpublished data). All these findings suggest that the substrate recognition by the enzyme is determined not only by the interaction of phosphate moiety with the enzyme P-loop, but also by favorable binding to some aromatic ring. In this paper we studied the phosphatase activity of Ltp1 on Fpr3, over-expressing the Ltp1 protein or its dominant negative mutant in *S. cerevisiae*. Moreover, we tested Fpr3 tyrosine phosphorylation level in a yeast mutant strain not expressing Ltp1. Site-directed mutagenesis demonstrated that Tyr 184 is the residue phosphorylated on in vivo Fpr3. We described also the marked activation of Ltp1 by adenine, resulting in changes in protein tyrosine phosphorylation in *S. cerevisiae* proteome in vivo. Finally, we determined in vivo the influence of tyrosine phosphorylation on Fpr3 localization.

Materials and methods

Strains, growth media, and plasmid. The yeast strains used in this study are described in Table 1. The parent strain is W303-1A *MAT*a *leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal.* Plasmid pAAH5 containing Ltp1 was constructed by a PCR experiment on total yeast DNA from W303 wild-type strain using the primers (5′-GGCCAAGCTTATGACAATTGAAAAACC-3′, 5′-GGCCAAGCTTCCGCACCATCATTAC-3′), which contain a *Hin*dIII site. The PCR fragment obtained was digested with *Hin*dIII and cloned in the *Hin*dIII site of the pAAH5 yeast expression vector [26] to obtain the recombinant plasmid pALtp1. This recombinant plasmid was used to transform the W303-1A strain in order to obtain the recombinant strain W303pALtp1. The cDNA expressing the Ltp1 mutant where the aspartate 123 was replaced by an alanine and the cDNA expressing the Fpr3 mutant where the tyrosine 184 was replaced by an alanine were performed on the pALtp1 and on the pyFpr3 plasmids using QuikChange Site-Directed Mutagenesis Kit (Stratagene) and, respectively, the primers:

5′-CTGTGCAGACCATTATTGAAGCTCCTTGGTATGGTGACATAC-3′,
5′-GTATGTCACCATACCAAGGAGCTTCAATAATGGTCTGCACAG-3′
5′-CGAAGATGAAGACGCTGACATCGCTGACAGTGAAGACTACG-3′,
5′-CGTAGTCTTCACTGTCAGCGATGTCAGCGTCTTCATCTTCG-3′.

Table 1
Yeast strains used

Strain	Genotype	Source and reference
W303-1A	MATa leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100, GAL SUC mal	[20]
W303pAAH5	W303-1A + pAAH5	[19]
W303pALtp1	W303-1A + pALtp1	This study
W303pALtp1D133A	W303-1A + pALtp1D133A	This study
W303pyFpr3	W303-lA + pyFpr3	[20]
JK93da	MATa his4 HMLa leu2-3,112 rme1 trp1 ura3-52	[24]
KDY86.6a	Δfpr3::URA3(JK93da)	[24]
KDY86.6apAAH5	KDY86.6a + pAAH5	[20]
KDY86.6apyEP351	Δfpr3::URA3(JK93da) + pyEP351	This study
KDY86.6apALtp1	KDY86.6a + pALtp1	This study
KDY86.6apyFpr3	Δfpr3::URA3(JK93da) + pyFpr3	This study
KDY86.6apyFpr3Y184A	Δfpr3::URA3(JK93da) + pyFpr3Y184A	This study
BY4743	MATa his3-1, leu2, lys2, ura3-52 Δltp1::KANmx	M. Tommasino

The two mutated cDNA obtained were called: pALtp1D133A and pyFpr3Y184A, respectively, and used for transformation of W303-1A to obtain the recombinant strains W303pALtp1 and W303pALtp1-D133A, respectively. The plasmid pyFpr3, used to obtain the over-expressing Fpr3 strain, is a gift from Thorner and co-workers [25].

Strain KDY86.6a lacking the Fpr3 gene and its isogenic wild-type strain JK93da were gifts from Heitman and co-workers [24]. The Ltp1 deletion strain BY4743 was a kind gift from M. Tommasino.

Cells were grown at 30 °C either in YP medium (1% yeast extract and 2% peptone (Biolife)) or in synthetic minimal medium (SD) containing 0.67% w/v YNB (Difco) and appropriate quantities of the "drop-out" amino acid–nucleotide mixture (Bio101). Two percentage w/v glucose was used as carbon sources. Solid media contained 2% w/v agar.

Expression of recombinant Ltp1 and Ltp1D133A in S. cerevisiae: immunoprecipitations and Western blot analysis. Expression of the active Ltp1 was assayed for its activity on *p*-nitrophenylphosphate (pNPP) as previously described [27]. For immunoprecipitation and Western blot analysis cells were grown to late exponential phase ($A_{600} = 1$). Cells were harvested by centrifugation and washed in water and finally the pellet was resuspended in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, and 1 mM sodium orthovanadate) with yeast protease inhibitors cocktail (Sigma). Cells were broken with glass beads in a Fastprep instrument (Savant) and protein extracts were clarified by centrifugation at 13,000 rpm for 10 min. For the immunoprecipitation 1 mg of total proteins was incubated with the specified antibodies in RIPA buffer and the immunocomplexes were collected on protein A–Sepharose (Amersham–Pharmacia Biotech), separated by gel electrophoresis (SDS–PAGE), and transferred to a polyvinylidene-difluoride membrane (PVDF) (Immobilon P; Millipore, Bedford, MA).

For Western blot an amount of 20–100 μg of total proteins was separated by SDS–PAGE and transferred to PVDF membrane. Immunoblot analysis was performed using either anti-PY20 antibodies (Santa Cruz Biotechnology) at a 1:1000 dilution, anti-phosphotyrosine monoclonal antibodies 4G10 (Upstaste Biotechnology) at a 1:10,000 dilution, rabbit polyclonal anti-Ltp1 antibody at 1:1000 dilution or rabbit polyclonal anti-Fpr3 antibody at 1:2000 dilution. The anti-Fpr3 antibodies were a gift from Prof. J. Thorner. Antibodies were detected by using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies. Immunoblots were visualized by using ECL reagents (Amersham–Pharmacia Biotech).

Isolation of yeast nuclei. To isolate yeast nuclei 500 ml of exponential growing cultures in YPD medium was collected, resuspended in 1 M sorbitol, 50 mM KPO_4 (pH 7.8), 0.5 mM PMSF, 10 mM $MgCl_2$, and 30 mM DTT to a density of 1×10^9 cells/ml, and incubated at 30 °C for 30 min. The cells were collected by centrifugation and resuspended in 1 M sorbitol, 25 mM KPO_4 + 25 mM sodium succinate (pH 5.5), 0.5 mM PMSF, 10 mM $MgCl_2$, and 2 mM DTT to a density of 5×10^9 cells/ml with 1 mg/ml Zymolyase. The spheroplasts are homogenized by 40 strokes with a Dounce homogenizer in Mes (pH 6.4), 10 mM $MgCl_2$, 0.5 mM PMSF, and 0.2% Triton X-100 [28]. The homogenate was centrifuged at 300*g* for 10 min to remove cell debris, nuclei, and mitochondria. The supernatant was centrifuged at 10,000*g* and the resulted supernatant was referred to as cytoplasmic fraction.

The pellet obtained from the fist centrifugation described was centrifuged for 35 min at 38,000*g* in the previously mentioned buffer containing 50% of Percoll. Nuclei form a predominant band near the bottom of the gradient.

Results and discussion

Previous studies reported that expression of fission yeast LMW-PTP Stp1 in *S. cerevisiae* has a strong influence on the tyrosine phosphorylation state of many different yeast proteins [19]. In order to obtain *S. cerevisiae* strains over-expressing the Ltp1 or its dominant negative Ltp1 mutant, we separately cloned the corresponding genes into the expression vector pAAH5 that allows constitutive expression. The corresponding recombinant plasmids, pALtp1, and pALtpD133A, were used to transform the W303 yeast strain obtaining the corresponding recombinant strains: W303pALtp1 and W303pALtp1D133A. The expression of the two proteins was assayed for the enzymatic activity using pNPP as substrate (Table 2) and Western blot analysis using polyclonal antibodies against Ltp1 (Fig. 1) to evaluate the expression of the wild-type and the dominant negative proteins. The recombinant strain over-expressing

Table 2
PTPase activity of clear lysates from Ltp1 and Ltp1D133A over-expressing strain in comparison to the control

Recombinant strains	U/ml[a]	mg/ml	U/mg
W303 + pAAH5	0.36	8.03	0.045
W303 + pALTP1	1.25	8.9	0.14
W303 + pALTP1D133A	0.36	10.7	0.034

[a] On pNPP as substrate.

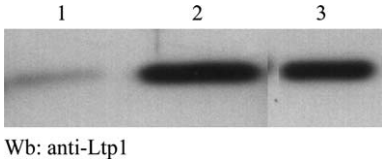

Fig. 1. Western blot analysis of Ltp1 and Ltp1 D133A overexpressing cells. Lysates from wild-type strain W303pAAH5 (1), W303pALtp1 (2), and W303pALtp1D133A (3) were analyzed by immunoblotting with anti-Ltp1 antibodies. 20 μg of total proteins were applied in each lane.

active Ltp1 showed an increase of the enzymatic activity in comparison with the control indicating the over-expression of the active Ltp1 protein. The two recombinant clones chosen showed high levels of recombinant protein expression, as reported in Fig. 1 in which two bands corresponding to the active (lane 2) and the dominant negative protein (lane 3) in the W303pALtp1 and W303pALtp1D133A strains, respectively, are shown. The two bands are evident with respect to the Ltp1 basal level of the empty vector control strain (lane 1).

Previous work from our laboratory indicated that anti-phosphotyrosine antibodies (PY20) are able to reveal phosphorylation on tyrosine in a *S. cerevisiae* strain, showing that many tyrosine-phosphorylated proteins are detectable in *S. cerevisiae*. We analyzed the modification of tyrosine phosphorylation in *S. cerevisiae* proteome in response to the over-expression of the LMW-PTP Stp1 or its dominant negative mutant. The tyrosine phosphorylation level of several proteins decreases upon active enzyme expression or increases in the yeast strain expressing the dominant negative protein. Recently we reported that blockage of the Ser/Thr kinase CK2 with specific inhibitor synergizes with the expression of Stp1 in inducing a severe growth defective phenotype and we presented in vivo evidence that immunophilin Fpr3 interacts with and is dephosphorylated by Stp1. We run a cell growth kinetic in the presence of the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB). Analyses of growth kinetics have shown that they are slightly affected by Stp1 expression alone but this effect is dramatically enhanced by concomitant addition of the specific CK2 inhibitor. This longer lag phase, observed within 8 h after TBB treatment, could correlate with a reduced level of Fpr3 phosphorylation [20].

Influence of Ltp1 on yeast tyrosine phosphorylation

Our first aim was to assay the capability of Ltp1 to dephosphorylate yeast substrates in vivo. In order to explore this aspect, the pattern of tyrosine-phosphorylated proteins has been analyzed in the recombinant *S. cerevisiae* strain W303pALtp1D133A (expressing the dominant negative mutant) in comparison with the control strain (harbouring the empty vector). To enhance the level of tyrosine-phosphorylated proteins we performed this experiment after an immunoprecipitation using anti-phosphotyrosine antibodies. Cell lysates from the W303pALtp1D133A strains and from the control strain were separately subjected to immunoprecipitation with anti-phosphotyrosine antibodies and immunoprecipitates were resolved by SDS–PAGE. Immunoblot using anti-phosphotyrosine antibodies was than performed. The results are reported in Fig. 2A where it is evident

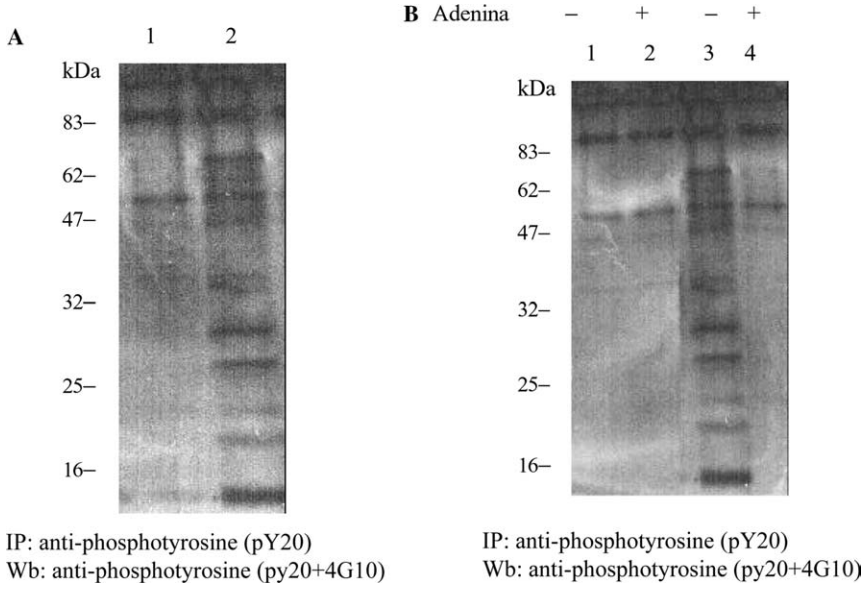

Fig. 2. The phosphorylation pattern and adenine treatment. Panel A. The phosphorylation pattern of W303pAAH5 (1) and W303pALtp1D133A (2). Phosphorylated proteins were detected by immunoprecipitation of 1 mg of total proteins with anti-phosphotyrosine antibodies. Panel B. Adenine effect on phosphorylated proteins. Lysates from W303pAAH5 (1, 2) and W303pALtp1D133A (3, 4) were treated with 5 mM adenine and then immunoprecipitated with antiphosphotyrosine antibodies.

that the number and the intensity of immunoreactive bands in W303pALtp1D133A strain (lane 2) is higher than in the empty vector control strain (lane 1). Expression of the negative mutant causes a dramatic increase in tyrosine phosphorylation level of many proteins with respect to the control strain, suggesting that Ltp1 is very active in dephosphorylating several different substrates. It should be noticed, in fact, that only when the dominant negative molecule is expressed, many different protein species are phosphorylated, while with basal wild-type Ltp1 expression the equilibrium is shifted towards dephosphorylation. On the other hand, it is well known that adenine activates more than 30-fold, the yeast LMW-PTP Ltp1. When W303pALtp1D133A and control strain lysates were incubated with 5 mM adenine, protein phosphorylation is dramatically reduced in the W303pALtp1D133A expressing strain lysates (Fig. 2B, lane 4), becoming very similar to that of the control strain. This observation indicates that adenine dependent activation of endogenous Ltp1 overcomes the effect of dominant negative expression. Adenine administration to control strain lysate does not show any effect, confirming that endogenous Ltp1 is very efficient in protein dephosphorylation. All these findings suggested that many yeast tyrosine-phosphorylated proteins can be dephosphorylated by Ltp1.

Ltp1 dephosphorylates Fpr3 in vivo

In a previous work we have shown that Tyr phosphorylated Fpr3 is an in vivo substrate of Stp1, the *S. pombe* enzyme 50% identical to Ltp1. To determine whether the activity of Ltp1 affected the tyrosine phosphorylation state of Fpr3, the immunoprecipitates with anti-phosphotyrosine antibodies shown in Fig. 2A were reprobed with anti-Fpr3 antibodies. The results are shown in Fig. 3 where, in the strain expressing the pALtp1D133A dominant negative mutant, a protein of apparent molecular mass of 70 kDa was recognized by anti-Fpr3 antibodies (Fig. 3, lane 2). Because Fpr3, as detected by immunoblotting with anti-Fpr3 antibodies, was evident only in the strain expressing the inactive dominant negative protein, we suggest that Ltp1 can directly dephosphorylate Fpr3. The experiment described above suggested us to check whether Ltp1 and Fpr3 interact in vivo with each other. This hypothesis was tested using two co-immunoprecipitation assays. Wild-type strain (expressing basal Ltp1 level) was used together with the dominant negative expressing mutant (in order to enhance tyrosine phosphorylation level) and with a strain lacking Ltp1 expression (in order to rule out the possibility of an aspecific interaction of Fpr3 with other endogenous proteins) in an immunoprecipitation assay using anti-Fpr3 antibodies. The immunoprecipitated proteins were separated on a SDS–PAGE and analyzed for the presence of Ltp1 using anti-Ltp1 antibodies. The immunoblot (Fig. 4A) revealed the presence of the Ltp1 protein in the immunoprecipitate of the strain expressing the dominant negative mutant Ltp1D133A (lane 1) and in the empty vector strain (lane 3) in which the endogenous Ltp1 is still present, but not in lane 2 in which the endogenous Ltp1 gene is deleted. These results indicate that in both the strains the native Ltp1 or its catalytically inactive mutant can capture Fpr3 as associated protein. To confirm the interaction between Ltp1 and Fpr3, the lysates from the strain expressing the dominant negative protein (W303pALtp1D133A) and from the W303pAAH5 strain expressing the wild-type Ltp1 were immunoprecipitated with anti-Ltp1 antibodies and the precipitates were immunoblotted with anti-Fpr3 antibodies. The formation of Ltp1–Fpr3 complex was again observed only in the strains expressing the dominant negative Ltp1 (Fig. 4B, lane 1) and in the empty vector wild-type strain (lane 3). As control strain we performed the same immunoprecipitation assay in

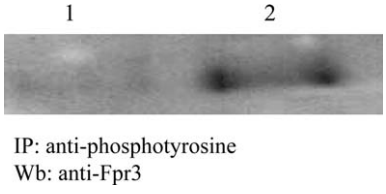

Fig. 3. Fpr3 co-immunoprecipitates with anti-phosphotyrosine from W303pALtp1D133A. The 70 kDa protein in the immunoprecipitate from W303pALtp1D133A overexpressing cells is the immunophilin Fpr3. The immunoblot of Fig. 2A was reprobed with anti-Fpr3 antibodies.

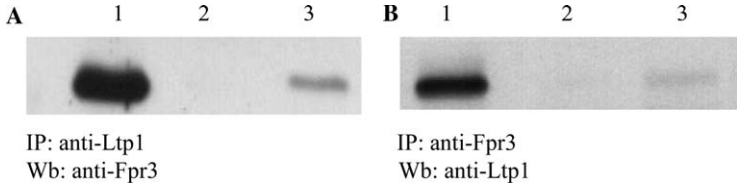

Fig. 4. Fpr3 and Ltp1 in vivo interaction. Panel A: total lysates from W303pALtp1D133A (1), BY4743ΔLTP1pAAH5 (2), W303pAAH5 (3) were immunoprecipitated with anti-Ltp1 antibodies and analyzed by immunoblotting with anti-Fpr3 antibodies. Panel B: total lysates from W303pALtp1D133A (1), KDY86.6apAAH5 (2), W303pAAH5 (3) were immunoprecipitated with anti-Fpr3 antibodies and analyzed by immunoblotting with anti-Ltp1 antibodies.

the *fpr3* null mutant (KDY86.6apAAH5) (lane 2). Therefore, we can conclude that Fpr3 in vivo interacts with Ltp1. To determine whether the activity of endogenous Ltp1 affects the tyrosine phosphorylation state of Fpr3 and to confirm that Ltp1 is active on Fpr3 in vivo, we compared the tyrosine phosphorylation level of yeast Fpr3 in strain not expressing Ltp1 (BY4743ΔLTP1), and in the control strain. Lysates from BY4743LTP1 and from the control strain BY4743 were immunoprecipitated using anti-phosphotyrosine antibodies and immunocomplexes were probed with anti-Fpr3 antibodies in a Western blot analysis. The results, showed in Fig. 5, demonstrate that the tyrosine phosphorylation level of Fpr3 is higher in BY4743ΔLTP1 strain (Fig. 5, lane 2), in comparison to the wild-type (Fig. 5, lane 1). These data confirm that Tyr phosphorylated Fpr3 is an in vivo substrate of Ltp1.

Tyr 184 is the tyrosine phosphorylation site in Fpr3 in vivo

Pinna and co-workers [29] with in vitro experiments identified Tyr 184 as the tyrosyl residue phosphorylated in Fpr3, residing in a highly acidic sequence recognized by CK2. This pleiotropic enzyme was shown to be responsible for Tyr phosphorylation of Fpr3 and to be able to phosphorylate in vitro a tyrosyl peptide reproducing the sequence around Tyr 184. In order to confirm that this phosphorylation occurs also in vivo, Tyr 184 was changed to Ala by site-directed mutagenesis on the plasmid YEP351 harbouring the wild-type Fpr3. The recombinant plasmid yEP351Fpr3Y184A was used to transform the KDY86.6a [Δfpr3::URA3(JK93da)] strain which contains disruption in *fpr3*. This strain was called KDY86.6apyFpr3Y184A. The expression of the protein was verified by Western blot analysis using polyclonal antibodies against Fpr3 (data not shown). The experiment was performed by immunoprecipitation with anti-phosphotyrosine antibodies on lysates from the KDY86.6a transformed either with the plasmid expressing the wild-type or the mutant Fpr3. The immunoprecipitates were recognized with anti-Fpr3 antibodies showing a sharp band only in the strain expressing the wild-type Fpr3 [KDY86.6apyFpr3] (Fig. 6, lane 1), confirming that Tyr 184 is the main target of phosphorylation in Fpr3. In order to verify if this residue is also important for Ltp1–Fpr3 association, lysates from the same yeast strains [KDY86.6apyFpr3Y184A, KDY86.6apyFpr3, and from the KDY86.6apyEP351 as control strain] were immunoprecipitated with anti-Ltp1 antibodies. Immunocomplexes were separated by SDS–PAGE and immunoblotted using anti-Fpr3 antibodies (Fig. 7A). Fpr3 protein is clearly visible only in lysates of the strain expressing wild-type Fpr3 (lane 2), while it is drastically reduced were the dominant negative is expressed and, as expected, totally absent in *fpr3* null mutant. In panel B the same immunoprecipitates where subjected to anti-Ltp1 immunoblot for normalization. These results demonstrate that Tyr 184 not only is the main site of phosphorylation of Fpr3, but is also responsible for Ltp1 binding.

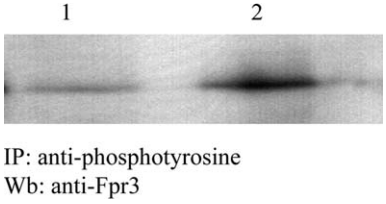

Fig. 5. Deletion of *ltp1* gene enhances Fpr3 phosphorylation. 1 mg of lysates from wild-type strain and BY4743ΔLTP1 strain were immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted with anti-Fpr3 antibodies.

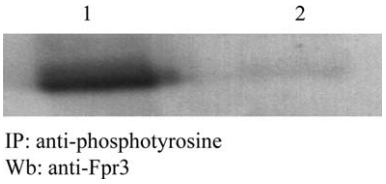

Fig. 6. Tyrosine 184 is the major phosphorylation site in vivo. Lysates from KDY86.6apyFpr3 (1) and KDY86.6apyFpr3Y184A (2) were immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted with anti-Fpr3 antibodies.

Localization of Fpr3 is independent from its phosphorylation on Tyr 184 residue

The subcellular compartment in which Fpr3 resides is still debated. To determine if tyrosine phosphorylation and dephosphorylation of Fpr3 might play a role in regulating its subcellular localization, we examined the localization of Fpr3 in the *fpr3* null mutant strain expressing either the wild-type Fpr3 (KDY86.6apyFpr3 strain) or the mutant protein Fpr3Y184A (KDY86.6apyFpr3Y184A strain). Lysates from these strains were subcellular fractionated as described in Materials and methods in order to purify the nuclei from the cytoplasmic fraction. The nuclear and cytoplasmatic fractions

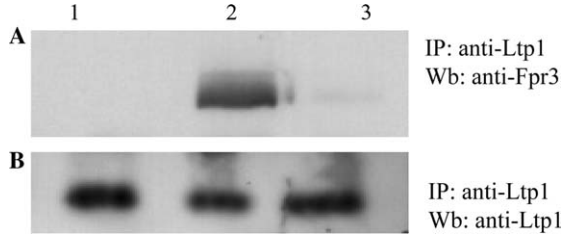

Fig. 7. The mutant Fpr3Y184A weakly interacts with Ltp1. 500 μg of total lysate from KDY86.6apyEP351 (1), KDY86.6apyFpr3 (2), and KDY86.6apyFpr3Y184A (3) were immunoprecipitated with anti-Ltp1 antibodies. The immunoprecipitates were subjected to western blot analysis using anti-Fpr3 antibodies (A) and anti-Ltp1 antibodies (B).

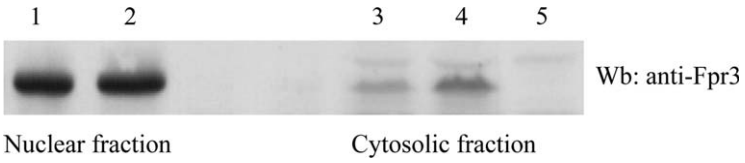

Fig. 8. Fpr3 cellular localization. 5 μg of nuclear fraction from KDY86.6apyFpr3 (1), KDY86.6apyFpr3Y184A (2) and 50 μg of cytoplasmic fraction from KDY86.6apyFpr3 (3), KDY86.6apyFpr3Y184A (4) were immunoblotted with anti-Fpr3 antibodies. Lane 5, 20 μg total lysates from KDY86.6ayEP351.

were separately by SDS–PAGE and analyzed by Western blot using anti-Fpr3 antibodies. A signal is predominantly detected in the nucleus in both the strains even when the mutant protein, in which the Tyr 184 is mutated to Ala, is over-expressed (Fig. 8). On the basis of these observations we propose that regulation of Fpr3 localization is independent from Tyr 184 phosphorylation. This is consistent with the fact that Fpr3 is a nuclear protein and that its tyrosine phosphorylation or dephosphorylation does not regulate its subcellular localization.

In conclusion, with this paper we demonstrate that Ltp1 is very active in dephosphorylating several different substrates in *S. cerevisiae*. In particular, we have shown that Fpr3 clearly interacts and is dephosphorylated in vivo by Ltp1. Using an appropriate mutant it was also possible to show that Tyr 184 is the main Fpr3 site of phosphorylation and is also responsible for Ltp1 binding and Ltp1 dependent dephosphorylation.

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