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The PPZ protein phosphatases are involved in the maintenance of osmotic stability of yeast cells

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We have recently reported the existence in the yeast *Saccharomyces cerevisiae* of a gene named *PPZ1*, encoding a novel Ser/Thr phosphatase characterized by a large, Ser-rich amino-terminal extension, and suggested the existence of a related gene product that could have overlapping functions. We have now amplified by polymerase chain reaction techniques a genomic fragment of about 600 bp corresponding to this second gene (*PPZ2*). This fragment hybridizes to an mRNA of about the same size as the *PPZ1* message but the amount of *PPZ2* mRNA peaks at the stationary phase, when almost no *PPZ1* mRNA is found. The PPZ2 fragment was interrupted in vitro and used to transform diploid heterozygous *ppz1 PPZ2* cells. Haploid cells carrying the double mutation *ppz1 ppz2* were unable to grow in the presence of 5 mM caffeine. However, the mutants did survive when osmotically stabilized in the presence of 1 M sorbitol. The evidence obtained suggests that PPZ1 and PPZ2 may be structurally and functionally related and points to an involvement of these phosphatases in functions related to the maintenance of cell integrity.

Ser/Thr protein phosphatase; Gene disruption; Osmotic stability; Cell lysis; *Saccharomyces cerevisiae*

1. INTRODUCTION

The presence of protein phosphatase activity in yeast was recognized a long time ago [1]. However, genes encoding putative Ser/Thr phosphatases were identified and cloned only recently [2–4]. Mammalian Ser/Thr protein phosphatases were classified on the basis of their enzymological properties into four different groups, namely types 1, 2A, 2B and 2C [5]. Yeast Ser/Thr protein phosphatases are very much related to their mammalian counterparts. This similarity was established on the basis of both their enzymological properties [6] and their primary structure. Yeast genes encoding the proteins homologous to the catalytic subunits of mammalian type 1 [3,7], 2A [8,9] and 2B [10,11] phosphatases were described and found to be involved in a large variety of very relevant cellular processes. However, it is now evident that phosphatases other than those previously recognized exist in eukaryotic cells. Examples are rabbit PPX and *Drosophila* PPV and PPY phosphatases [12,13]. In an attempt to identify novel phosphatases in yeast and to study their cellular functions, we performed genomic DNA amplification experiments based on the existence of conserved sequences found in Ser/Thr phosphatases in yeast and higher eukaryotic cells. As a result, we have recently cloned two genes encoding nodel types of phosphatases, namely *PPG* [14] and *PPZ1* [15]. The PPZ1 protein was found to be a large polypeptide (692 residues) containing an unusually long amino-terminal extension, very rich in Ser and Thr residues. Interestingly, disruption of the *PPZ1* gene did not result in evident phenotypic changes. As an explanation, we suggested the existence of a second, related gene with overlapping functions. We report here the existence of such a gene, namely *PPZ2*, and we present evidence for the fact that the PPZ1/PPZ2 proteins are involved in the maintenance of the osmotic stability of yeast cells.

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2. MATERIALS AND METHODS

2.1. *Materials*

Oligonucleotides 5′-GG*AATT*CCGATTATTCAAATTTGCCAT-3′ (PPZ2-A) and 5′-GG*AATT*CCACACTAAATCGAATCCG-3′ (PPZ2-B) were used for amplification of a fragment of gene *PPZ2* (Italicized sequences correspond to *Eco*RI restriction sites added to facilitate subsequent cloning). *Thermus aquaticus* DNA polymerase, restriction enzymes and the nonradioactive DNA detection system were purchased from Boehringer Mannheim.

2.2. *Strain and media*

S. cerevisiae M5 (*MATa/MATα, PPZ1/PPZ1 PPZ2/PPZ2* and homozygous for *leu2-3 ura 3-52 trp1*) and JA14 (*MATa/MATα, PPZ1/ppz1::URA3 PPZ2/PPZ2*) strains were used. Yeast were grown at 30°C in YPD or SD synthetic medium [16]. *E. coli* cells were grown at 37°C in LB medium containing 50 μg/ml ampicillin for plasmid selection.

2.3. *Recombinant DNA techniques and standard genetic methods*

Bacterial cells were transformed as described previously [17]. Yeast

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cells were transformed by using a modification of the lithium acetate method [18]. Genomic DNA was prepared as described [16]. DNA probes were labeled by the random priming method using either [^{32}P]dCTP or digoxigenin-labeled dUTP. Standard DNA recombinant techniques were performed essentially as described [19]. Tetrad analysis and scoring of markers were performed by standard methods [16].

2.4. *Genomic DNA amplification*

Genomic DNA (0.2 μg) from strain M5 was amplified in a 100 μl reaction using oligonucleotides PPZ2-A and PPZ2-B (0.5 μM each) in the presence of 2 mM magnesium chloride. Amplification was carried out for 30 cycles (2 min at 94°C, 2 min at 45°C and 2 min at 72°C) and the reaction mixture purified, digested with *Eco*RI and cloned into the *Eco*RI site of plasmid Bluescript SK(+) to give pACG-2. The nucleotide sequence of the cloned DNA was determined by the dideoxynucleotide chain termination method [20] using fluorescent primers in an Applied Biosystems 373A automatic DNA sequencer.

2.5. *Southern and Northern blot analysis*

For Southern blot experiments genomic DNA (10 μg) was digested with different restriction enzymes, electrophoresed on 0.7% agarose gels and transferred to nylon membranes under vacuum. DNA was cross-linked to the membranes using a UV Stratalinker (Stratagene). Hybridization was performed at 65°C in 6 × SSC (1 × SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's solution (1 × is 0.1% (w/v) Ficoll 400, 0.1% (w/v) polyvinylpirrolidone, 0.1% bovine serum albumin and 0.001% SDS), 0.5% SDS and 150 μg/ml freshly denatured salmon sperm DNA. The ^{32}P-labeled amplification fragment was used as probe (10^6 cpm/ml). Filters were washed at 65°C in 0.1 × SSC, 0.1% SDS, unless otherwise stated.

Total RNA was prepared as described [21], electrophoresed on 0.7% agarose-formaldehyde gels (40 μg per lane) and transferred to nylon membranes under vacuum. Membranes were hybridized at 42°C in the presence of 50% (v/v) formamide and 10^6 cpm/ml of the appropriate ^{32}P-labeled DNA probe and washed in 0.1 × SSC, 0.1% SDS as stated.

2.6. *Gene disruption methods*

The one-step gene disruption method [22] was used. Plasmid pACG-2 was digested with *Bgl*II and ligated with a *Bgl*II–*Bam*HI 0.85 kbp fragment containing the gene *TRP1* [15]. The resulting plasmid was cleaved with *Cla*I and *Sac*I (both sites present in the poly-linker of plasmid Bluescript SK) to give a linear 1.48 kbp DNA fragment that was gel purified and used for transformation.

2.7. *Other methods*

For measuring release of alkaline phosphatase activity from the cells, aliquots of the culture were centrifuged for 2 min in a microfuge and the medium was saved and stored at −20°C. Alkaline phosphatase activity in the medium was measured at 37°C using *p*-nitrophenyl phosphate as substrate in a Cobas Bio autoanalyzer (Roche).

Glucose levels in the medium were measured using a Gluco-quant kit from Boehringer Mannheim, adapted for a Cobas Bio autoanalyzer. Qualitative determination of glycogen accumulation was estimated by inverting plates containing patches of cells over iodine crystals.

Heat-shock sensitivity was tested by streaking strains onto YPD plates (prewarmed at 55°C). Plates were incubated at 55°C for 45 min and then transferred at 30°C for two days. A *bcy*$^-$ strain was used as control in these experiments.

3. RESULTS AND DISCUSSION

We have recently reported the existence in *S. cerevisiae* of a gene encoding a putative protein phosphatase, PPZ1, structurally related to the type 1 Ser/Thr phosphatases. An unusual feature of the PPZ1 gene

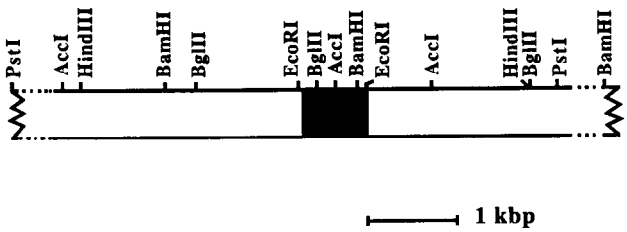

Fig. 1. Composite restriction site map of the *PPZ2* locus. The *Eco*RI–*Eco*RI amplification fragment was used to probe genomic Southern blots. DNA from strain M5 was digested with different enzymes (or combination of enzymes) and the information obtained used to generate the restriction site map. The black box represents the location of the probe. The most 5′ *Pst*I site is located approximately at 4.8 kbp from the nearest *Acc*I site shown. The most 3′ *Bam*HI site is found at about 11 kbp from the nearest *Bam*HI site shown.

product was the existence of a large Ser-rich amino-terminal extension, not found in other phosphatases [15]. Disruption of the *PPZ1* gene did not result in any evident phenotypic change. Southern blot experiments performed at low stringency in our laboratory suggested the possibility of a second, related gene the product of which might have overlapping functions. The existence of this second gene (named *PPZ2*) was consistent with the finding of a 666 bp DNA fragment encoding a polypeptide related to sequences found in type 1 phosphatases [23]. This PPZ2 sequence could be aligned from residue 389 to 610 of our PPZ1 protein (93% identity) and, although initially isolated from a commercial rabbit brain cDNA library, it was subsequently identified as of yeast origin [23].

Since the sequence of the above-mentioned PPZ2 fragment was available, we designed oligonucleotides PPZ2-A and PPZ2-B to selectively amplify most of this sequence from yeast genomic DNA by polymerase chain reaction techniques. A fragment of the expected size (about 630 bp) was amplified and sequenced (not shown). Sequence analysis of the 596 bp encompassed by the oligonucleotides revealed only three nucleotide differences (nucleotides 405, 420 and 492) when compared with the published sequence [23]. These changes did not result in changes of amino acids and could be attributed to differences between yeast strains. To characterize the genomic locus of *PPZ2*, we performed Southern blot experiments after digestion of yeast genomic DNA with different restriction enzymes. The genomic map constructed from different experiments is presented in Fig. 1. This map is consistent with the additional signals observed at low stringency using a *PPZ1* probe (not shown).

The availability of a *PPZ2* probe allowed us to study different aspects of this gene in comparison to *PPZ1*. Thus, Northern blot experiments were performed using total RNA from cultures at different phases of growth (Fig. 2). These experiments resulted in two interesting observations. First, the *PPZ2* probe hybridized with an

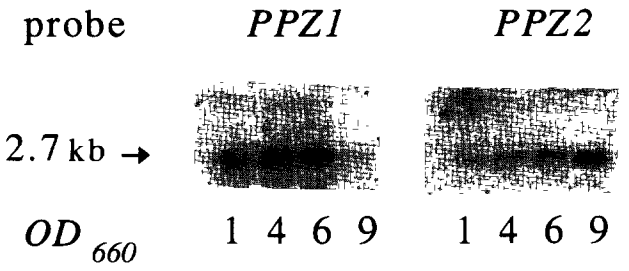

Fig. 2. Northern blot analysis of PPZ2 mRNA. Cultures of strain M5 were grown and samples taken at different times. Total RNA was prepared, electrophoresed (40 μg/lane) and transferred to membranes. Filters were probed with a ^{32}P-labeled *Pst*I–*Acc*I 2.3 kbp DNA fragment containing most of the coding region of the *PPZ1* gene [15] or the 0.63 kbp amplification fragment corresponding to gene *PPZ2*. Washing was performed in 0.1 × SSC, 0.1% SDS at 65°C (*PPZ1* probe) or 55°C (*PPZ2* probe).

mRNA of about the same size as that observed for PPZ1. Despite of the fact that only a limited amount of the PPZ2 sequence was available, this could indicate that the PPZ1 and PPZ2 proteins might be related proteins of roughly similar size (see below). Second, the *PPZ1* and *PPZ2* genes are expressed differently during the growth of the culture. As shown in Fig. 2, the pattern of mRNA level for *PPZ1* is as previously reported [15], that is, *PPZ1* mRNA peaks at the medium–late exponential phase and almost disappears at saturation. On the contrary, *PPZ2* mRNA increases during the culture and reaches its highest levels in saturated cultures, when glucose in the medium is exhausted.

Our main interest was to learn whether the existence of a gene product similar to the PPZ1 protein was the reason for the lack of evident phenotypic changes observed in *ppz1*$^-$ cells. Although we have not cloned the entire *PPZ2* open reading frame, we considered that the cloned amplification fragment was enough to undertake gene disruption experiments. To this purpose we introduced a yeast *TRP1* gene into the *Bgl*II site contained in the PPZ2 fragment and transformed diploid JA14 cells (*PPZ1/ppz1*::*URA3 PPZ2/PPZ2*) with this construction (Fig. 3). Stable uracil and tryptophan prototrophic colonies were selected and tested for disruption of *PPZ2* by Southern blot analysis (Fig. 3). Several positive cells were sporulated and tetrads dissected. This approach provided us with wild type cells, cells carrying a disruption only in gene *PPZ1*, only in gene *PPZ2*, and in both genes. The existence of the predicted mutations in the haploid cells was confirmed by Southern blot using *PPZ1* and *PPZ2* probes (not shown).

In our search for phenotypes associated with the *ppz1/ppz2* mutation we have found that the simultaneous interruption of both genes renders the cells hypersensitive to caffeine. As observed in Fig. 4, *ppz1*::*URA3 ppz2*::*TRP1* cells cannot grow in YPD supplemented with 5 mM caffeine but growth can be restored by transformation with plasmid pACG1, a multicopy plasmid carrying the entire *PPZ1* gene [15]. Lack of the PPZ1 protein appears to be more detrimental for the cells than disruption of *PPZ2*, since *ppz2*$^-$ cells can still grow in 10 mM caffeine whereas *ppz1*$^-$ cells cannot (wild type cells can survive at least 15 mM caffeine). It is worth noting that the caffeine hypersensitivity observed in *ppz1 ppz2* cells is also characteristic of mutations in the genes encoding the yeast homologue of mammalian type 1 phosphatase [3], which are structurally related to the PPZ1/PPZ2 proteins. However, it must be kept in mind that, although perhaps the type 1 phosphatases and the PPZ1/PPZ2 proteins might share some common activities within the cell, disruption of *DIS2S1*, the sole type 1 phosphatase gene in *S. cerevisiae*, is lethal [24,25] and, therefore, these proteins cannot have fully overlapping functions.

Although the effects of caffeine are pleiotropic, this compound is a well known inhibitor of phosphod-

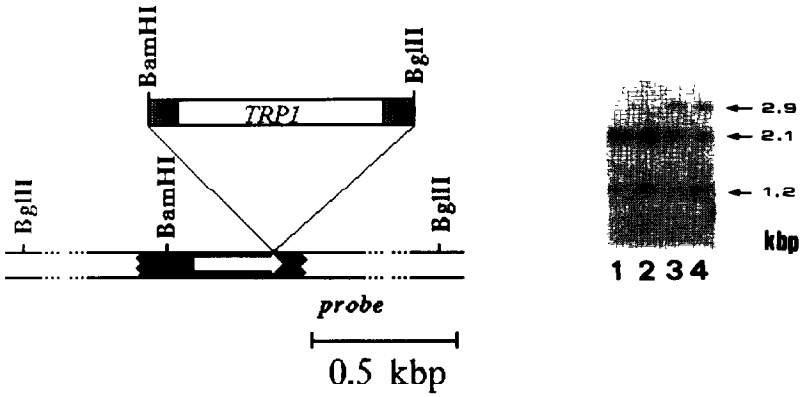

Fig.3. Disruption of gene *PPZ2*. The *S. cerevisiae* gene *TRP1* was inserted into the *Bgl*II site present in the *PPZ2* amplification fragment (see section 2). The arrow indicates the direction of translation. A linearized 1.48 kbp *Sac*I–*Cla*I fragment was used to transform the diploid strain JA-14 (which is heterozygous for the *ppz1*::*URA3* mutation). Genomic DNA was prepared from JA-14 and trp$^+$ cells, digested with *Bgl*II, transferred to membranes and probed with the 0.63 kbp *PPZ2* amplification fragment. Lanes 1 and 2 correspond to strain JA-14. Lanes 3 and 4 correspond to transformed cells carrying the *ppz2*::*TRP1* disruption. Figures on the right correspond to the estimated size of the fragments hybridizing with the probe.

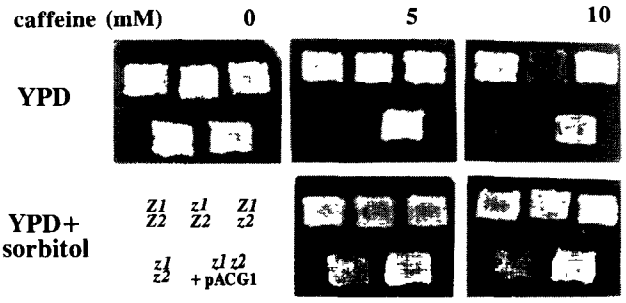

Fig. 4. Sensitivity to caffeine of *ppz1*, *ppz2* and *ppz1 ppz2* mutants. Patches of wild-type, *ppz1 PPZ2*, *PPZ1 ppz2*, *ppz1*, *ppz2* and *ppz1 ppz2* cells containing plasmid pACG1 (a multicopy plasmid containing the entire *PPZ1* gene) were streaked on YPD or YPD supplemented with 1 M sorbitol plates, as indicated. Cells were incubated for 2–3 days at 30°C in the absence or the presence of different concentrations of caffeine.

iesterase and, therefore, it provokes an increase in the intracellular levels of cAMP and a concomitant activation of the cAMP-dependent protein kinase. In fact, caffeine hypersensitivity is also a characteristic phenotype of mutants having an increased cAMP-dependent protein kinase activity, as it is the case of *bcy*$^-$ cells [26]. *bcy*$^-$ mutants are heat-shock sensitive, as it has also been reported for other mutants in the RAS/cAMP pathway. Interestingly enough, the *ppz1 ppz2* mutants also display the heat shock-sensitive phenotype (not shown). These similarities might suggest that the lack of PPZ1/PPZ2 provokes the same effects as the activation of the cAMP-dependent protein kinase pathway and might be indicative for the fact that the PPZ1/PPZ2 phosphatases can counteract the biological effects of cAMP-dependent phosphorylation. However, it is important to note that even if this hypothesis proves to be correct, the counteraction is not absolute. For instance, we have been unable to observe any defect in glycogen accumulation in *ppz1 ppz2* mutants, a phenotype observed as a result of the activation of the cAMP dependent pathway. These results could be explained if we assume that PPZ1/PPZ2 act downstream from the cAMP-dependent protein kinase and that only a subset of the substrates of this kinase are also substrates for PPZ phosphatases.

A most interesting result is the fact that growth of *ppz1 ppz2* cells in the presence of caffeine can be restored if 1 M sorbitol, an osmotic stabilizer, is added to the plates (Fig. 4). Restoration of growth is also achieved when YPD plates are supplemented with the osmotically active compounds sodium chloride (0.5 M) or galactose (1 M) (data not shown). These results suggest that the lack of growth observed in the presence of caffeine could be attributed to a failure to maintain the cell integrity. This hypothesis is reinforced by the fact that, as shown in Fig. 5, exposure of liquid cultures of *ppz1 ppz2* cells to 5 mM caffeine results in the release of the cytoplasmic enzyme alkaline phosphatase to the medium. In addition, release of alkaline phosphatase activity is suppressed in the presence of 1 M sorbitol. Overexpression of *PPZ1* as a multicopy plasmid also prevents the release of alkaline phosphatase activity in *ppz1 ppz2* cells (not shown).

Therefore, the results presented in this paper represent the first report of a protein phosphatase involved in the maintenance of the osmotic integrity of yeast cells. This is a very interesting discovery since a role for protein phosphorylation in the maintenance of cell integrity was only very recently postulated by several laboratories. For instance, osmotic stability defects are also characteristic for *S. cerevisiae* strains lacking functional versions of the *PKC1* gene product, the yeast homologue of mammalian protein kinase C [27–29]. Several genes encoding downstream components of a pathway that bifurcates after PKC1 have been identified very recently (see [30] for a review). This pathway consists of several protein kinases and its activation would result in the final activation of the *MPK1* gene product, a protein kinase homologue of the mitogen-activated protein kinase (MAP-kinase) found in higher eukaryotic cells. Interestingly enough, mutations in components of this pathway, as it is the case of the genes *BCK1/SLK1* [26,31] and *MPK1* [32] result in a temperature-sensitive cell lysis defect which is also suppressed by osmotic stabilizing agents. In fact, the gene *MPK1* is identical to gene *SLT2*, which was previously identified as a multicopy suppressor of the lytic phenotype of *S. cerevisiae lyt2* mutants [33].

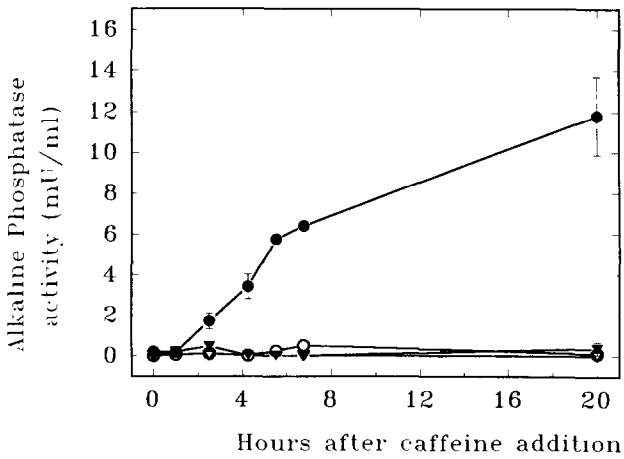

Fig. 5. Release of alkaline phosphatase in wild-type and *ppz1 ppz2* cells. Wild type haploid (▽, ▼) or *ppz1 ppz2* mutant cells (○, ●) were grown to OD = 1 in YPD (▼, ●) or YPD supplemented with 1 M sorbitol (▽, ○) at 30°C. The cultures were then made 5 mM caffeine and incubation was resumed. Control cultures received at the same time the same volume of sterile water. Aliquots of the cultures were taken at different times and the cells removed by filtration. The release to the medium of the cytoplasmic enzyme alkaline phosphatase was measured spectrophotometrically. Each value represents the difference between the enzyme activity in the presence or the absence of caffeine. Data are mean ± S.E.M. from 3–5 independent experiments.

From the data presented here one might expect that the functions of PPZ1/PPZ2 may be somehow related to the PKC1/MPK1 pathway. This idea is supported by two facts. First, mutations in the *PKC1/MPK1* pathway results in hypersensitivity to caffeine, as reported in this paper for *ppz1 ppz2* mutants. Thus, it has been reported that a *bck1*$^-$ strain fails to grow at 3 mM caffeine [26] and the same phenotype was observed in our laboratory in *mpk1* deletion mutants. Second, the gene *PPZ2* was very recently isolated as a dosage-dependent suppressor of the *mpk1* deletion and found, as expected, to code for a Ser-rich protein, very similar in size and structure to PPZ1 (D.E. Levin, personal communication). The idea that the PPZ1/PPZ2 phosphatases might interact with both the RAS/cAMP- and the PKC1/MPK1-mediated signalling systems, would be very attractive since it would establish a link between both pathways. In addition, it would now be necessary to address the molecular basis (role in cell wall synthesis, cytoskeleton assembly, ...) through which these phosphatases are relevant in the maintenance of cell integrity.

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