Molecular Characterization of Ypi1, a Novel Saccharomyces cerevisiae Type 1 Protein Phosphatase Inhibitor*

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The Saccharomyces cerevisiae open reading frame YFR003c encodes a small (155-amino acid) hydrophilic protein that we identified as a novel, heat-stable inhibitor of type 1 protein phosphatase (Ypi1). Ypi1 interacts physically in vitro with both Glc7 and Ppz1 phosphatase catalytic subunits, as shown by pull-down assays. Ypi1 inhibits Glc7 but appears to be less effective toward Ppz1 phosphatase activity under the conditions tested. Ypi1 contains a 48RHNVRW53 sequence, which resembles the characteristic consensus PP1 phosphatase binding motif. A W53A mutation within this motif abolishes both binding to and inhibition of Glc7 and Ppz1 phosphatases. Deletion of YPI1 is lethal, suggesting a relevant role of the inhibitor in yeast physiology. Cells overexpressing Ypi1 display a number of phenotypes consistent with an inhibitory role of this protein on Glc7, such as decreased glycogen content and an increased growth defect in a slt2/mpk1 mitogen-activated protein kinase-deficient background. Taking together, these results define Ypi1 as the first inhibitory subunit of Glc7 identified in budding yeast.

In eukaryotic organisms, protein phosphatases play a key role in the control and integration of cellular physiology. Among them, type 1 protein phosphatases (PP1) regulate a great variety of physiological processes in the cell such as carbohydrate and lipid metabolism, protein synthesis, and cell cycle progression (1–3).

The PP1 catalytic subunit (PP1c) is highly conserved throughout evolution. In most eukaryotes, several isoforms have been described (i.e. four in mammals), although in the yeast Saccharomyces cerevisiae only one PP1c is present, named Glc7, which is essential for cell viability (4, 5). Similar to its mammalian counterpart, Glc7 participates in the regulation of many different cellular processes such as glycogen metabolism, glucose repression, iron homeostasis, mitosis, meiosis, sporulation, vacuole fusion, endocytosis, polyadenylation termination, and cell wall integrity (6–14).

PP1c functional versatility can be achieved due to the existence of several regulatory subunits that act either targeting PP1c to different subcellular compartments and/or substrates, conferring substrate specificity or modulating enzymatic activity (15, 16). To date, more than 45 bona fide or putative PP1c-regulating subunits have been defined in higher eukaryotes (15–17). These subunits are structurally quite different, but almost all of them present a consensus binding motif (R/K)(V/I)X(F/W) necessary for PP1c regulation, which can also account for the mutually exclusive binding of the different subunits to PP1c (15–20).

PP1c activity is essential but must be tightly controlled, since overexpression or hyperactivation of PP1c phosphatase is also deleterious to the cell. Consequently, a large number of physiological inhibitors of PP1c have been identified in higher eukaryotes (15–17, 21). Among them, inhibitor-1 and inhibitor-2 are of special interest because they represent two different ways of inhibiting PP1c phosphatase activity. Inhibitor-1 and its structural homologue DARPP-32 require phosphorylation by the cAMP-dependent protein kinase A to gain PP1c-inhibitory capacity. In contrast, inhibitor-2 inhibits PP1c only in its dephosphorylated form (16, 21–23). Most of the PP1c inhibitors present the consensus PP1c binding motif described above, but several reports have shown that the association of inhibitory proteins to PP1c may involve additional contacts (23–26).

Mammalian inhibitor-1 and inhibitor-2 can also inhibit the yeast PP1c phosphatase Glc7 (25, 27, 28). However, no yeast homologue of inhibitor-1 has been described yet, and the yeast homologue of mammalian inhibitor-2, Glc8 (29, functions), as an activator rather than as an inhibitor of Glc7 (30). Recently, in a two-hybrid screening of a human brain cDNA library searching for potential mammalian PP1e regulatory proteins, a novel PP1 inhibitor, namely inhibitor-3, was identified. This protein shared 21% identity with a protein of unknown function encoded by the yeast YFR003c open reading frame (31). It was also demonstrated by two-hybrid analysis that the Yfr003c protein could interact with Glc7 (32–34). Therefore, this protein could be a good candidate for an endogenous inhibitor of Glc7 phosphatase activity.

Ppz1 and Ppz2 are PP1-related phosphatases involved in saline tolerance, cell wall integrity, cell cycle progression, and protein translation regulation, and, very recently, they have also been related to regulation of K+ and pH homeostasis (35, 36). Among them, Ppz1 appears to be more relevant than Ppz2 in regulating the functions mentioned above (35). Recent results indicate that Ppz phosphatases and Glc7 might have overlapping functions to some extent and that Ppz1 shares a subset of Glc7 regulatory subunits to fulfill its function (37). Interestingly, the Yfr003c protein has also been reported to interact with Ppz1 in a two-hybrid analysis (37). In this sense,
Yfr003c could also be a good candidate for an inhibitor of Ppz1 phosphatase activity in the same way as Hal3, a specific inhibitor of this type of phosphatases (38), which appears to regulate all known functions of Ppz1 (35).

In this report, we provide both in vitro and in vivo evidence demonstrating that the protein encoded by YFR003c is an inhibitor of the type 1 protein phosphatase Glc7 and, to some extent, perhaps of Ppz1. Hence, we propose the name Ypi1 (for yeast phosphatase inhibitor 1) for this protein.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**—Escherichia coli DH5α was used as the recipient cell for all plasmids and constructions. Yeast strains used in this work are listed in Table I. The ypi1Δ heterozygous null mutant was constructed using a diploid strain, S5 (see Table I), by a one-step short flanking kanamycin disruption method (39). The disruption cassette was generated by PCR using as template plasmid pFA6a-kanMX4 and primers YFRdel-1 and YFRdel-2 (see below). In this way, we disrupted by homologous recombination the complete YPI1 (YFR003c) open reading frame (from +1 ATG to the stop codon) in one of the two wild type alleles of the diploid. Mutants were confirmed by genomic PCR using specific wild type allele (oligonucleotides YFR-1 and YFR-2) and for the disrupted allele (oligonucleotides YFRPR-1, outside the disruption cassette, and YFRdel-2, inside the KanMX4 selection marker). Tetrad analysis was performed by standard methods, and the presence of the disruption cassette in the viable spore progeny was scored by its associated phenotype (growth in the YPD containing 200 μg/ml Geneticin plates).

Standard methods for genetic analysis and transformation were used. Yeast cultures were grown in rich medium (YPD) or synthetic complete (SC) medium lacking appropriate supplements to maintain plasmids (40), containing the indicated carbon sources. \( \text{slt}2/\text{mwp1} \) mutants were selected on plates containing 1 M sorbitol.

**Oligonucleotides**—The following oligonucleotides were used in the present study: YFR-1, GTTGGATTACATTAGGGTGGGAAAATACCTG; YFR-2, TTTTGGCAGAAGGACGTGAGTCTT; YFRPR-1, CGGCA-ATTCTGCGTACAAGGATGGAGC; YFRdel-1, TGCCGCAAGTT-GTGGAGCTCGCTGAG; YFRdel-2, TGGCGCTCATATTGTGGTTTGTGAACACCGCTAAGCAGTGATTTAGGTCTTCG; YFR53A-1, CTACAAGGCAAATAGGAAGATGTGGAGGGTTAGG; YFR3A-2, CATGATGCATATGGGTTTTCATAGAATGAGTAGTAGTAAG; YFR53A-3, GATGGGTATAGGAAGATGTGGAGGGTTAGG; YFR53A-4, GATGGGTATAGGAAGATGTGGAGGGTTAGG; YFR53A-5, GATGGGTATAGGAAGATGTGGAGGGTTAGG; YFR53A-6, GATGGGTATAGGAAGATGTGGAGGGTTAGG.

**Protein Phosphatase Assays**—Protein phosphatase activity using p-nitrophenylphosphate as substrate was determined essentially as described in Ref. 39. The reaction buffer was 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 150 mM NaCl, 10% glycerol, and 100 μg/ml phosphatase inhibitor mixture (Roche Applied Science). This buffer was made 2 mM MnCl₂ when purifying GST-Glc7 and GST-Ppz1-1344 fusion proteins. Cells were disrupted by sonication, and the fusion proteins were purified by passing the extracts through a 1-ml bed volume of glutathione-Sepharose columns (Amersham Biosciences). To remove the GST moiety from GST fusions to Ypi1 and Ypi1W53A, the fusion proteins bound to the glutathione-Sepharose beads were treated with PreScission Protease (Amersham Biosciences) during 4–5 h at 4 °C following the manufacturer's instructions. GST-Glc7, GST-Ppz1-1344, GST-Hal3, and GST proteins were eluted from the column with 10 mM glutathione. Samples were stored at −80 °C.

**Pull-down Assays and Immunoblot Analysis**—Preparation of yeast protein extracts for pull-down assays was essentially as described previously (10). Harvested bacterial cells were grown until exponential phase (approximately 0.3 optical density at 600nm) in LB medium containing 4% glucose as carbon source, and shifted to a medium containing 0.05% glucose, the reaction was stopped by adding 1% Triton X-100, 10 mM MOPS, pH 7.0, and 0.2 mM dithiothreitol. Samples were incubated for 10 min at 30 °C, and then the reaction was stopped by adding 1% Triton X-100 (final concentration). For phosphatase inhibition assays, different amounts of the purified inhibitors were incubated with the purified phosphatases during 5 min at 30 °C, prior to the addition of p-nitrophenylphosphate.
When potential phosphatase inhibitors were assayed, different amounts of the purified inhibitors were incubated with the purified phosphatases during 5 min at 30 °C, prior to the addition of the yeast crude extract.

**Measurement of Glycogen Content**—Wild type strain JA100 containing plasmids pWS93 or pWS-Ypi1 were grown on YPD until the indicated absorbance at 660 nm and then ~200 mg (wet weight) of fresh cells were collected by filtration. Cells were disrupted, and glycogen was measured as described.

**Fig. 1.** Ypi1 (Yfr003c) is conserved throughout all eukaryotes. A, phylogenetic tree of Ypi1 protein homologues. Multiple alignments and a phylogenetic tree of all of the Ypi1 homologues was generated using the Genebee service (available on the World Wide Web at www.genebee.msu.su). The name of the corresponding protein is written on the right of the respective organism. The numbers given above the branches indicate the percentages of 100 bootstrap resampled data sets supporting the clade to the right of the branch. B, a BLAST analysis (65) was performed to identify proteins having significant homology with the central domain of Ypi1 protein. GenBank accession numbers for the corresponding proteins are shown on the left. Sc, S. cerevisiae; Sp, Schizosaccharomyces pombe; Ne, Neurospora crassa; Ca, Candida albicans; Pf, Plasmodium falciparum; Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Ag, Anopheles gambiae; At, Arabidopsis thaliana; Mm, Mus musculus; Hs, Homo sapiens. The numbers indicate the positions in the corresponding protein where the homologous sequence begins and ends. Protein length in amino acids is indicated on the right. The Glc7 binding motif is indicated with a solid line, and the tryptophan residue involved in Glc7 binding is denoted in boldface type.

PAGE and immunoblot. When potential phosphatase inhibitors were assayed, different amounts of the purified inhibitors were incubated with the purified phosphatases during 5 min at 30 °C, prior to the addition of the yeast crude extract.
measured essentially as in Ref. 47. Glucose released by glycogen hydrolysis was measured using a glucose-oxidase-based commercial kit.

Phenotypic Analyses and Other Techniques—The effect of the overexpression of Ypi1 was monitored on plates by "drop tests" as previously described (48). Briefly, cells were grown on SC medium lacking uracil for 48 h, and absorbance at 660 nm was measured and adjusted to 0.05. Serial dilutions (1:5) were made, and 3 μl of each dilution was spotted on the indicated culture media.

To monitor recovery from α-factor arrest, strain JA110 (sit4Δ) was transformed with plasmid pWS93 or pWS-Ypi1, and cells were grown

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**Fig. 2. Ypi1 interacts physically with Glc7 and Ppz1 protein phosphatases.** A, pull-down assays between Glc7 phosphatase and different forms of Ypi1. Bacterial crude extracts (500 μg) prepared from E. coli DH5α expressing GST-Glc7 or GST were used to purify these proteins with GSH-Sepharose. Yeast crude extracts (500 μg), prepared from FY250 cells growing in glucose and expressing HA-Ypi1 (plasmid pWS-Ypi1) or HA-Ypi1W53A (plasmid pWS-Ypi1W53A), were added to the purified GST-Glc7 or GST proteins. Proteins that co-purified with GST-Glc7 and GST were analyzed by SDS-PAGE and immunodetected with anti-HA monoclonal (upper panel) or anti-GST-polyclonal (lower panel) antibodies. Proteins in the yeast crude extracts (1 μg) were also immunodetected with anti-HA antibodies (middle panel). Size standards are indicated in kDa.

**Fig. 3. Inhibition of Glc7 and Ppz1 phosphatase activities by Ypi1.** A and C, p-nitrophenyl phosphate dephosphorylation assays were carried out as described under "Materials and Methods." 1.8 μg of purified GST-Glc7 (A) or GST-Ppz11–344 (C) were incubated in the presence of p-nitrophenylphosphate (40 mM) as substrate. Increasing amounts of purified Ypi1 (diamonds), Ypi1W53A (squares), GST-Hal3 (triangles), or GST (circles) proteins were added to the reaction mixture. Values are means ± S.D. for at least three different assays, expressed as percentage of phosphatase activity with respect to control without inhibitors. B and D, HA-Reg11–443 dephosphorylation assays. MCY3000 cells expressing HA-Reg11–443 were grown in high glucose (4%) medium and shifted 20 min to low glucose medium (0.05%). Crude extracts were then obtained, and 1 μg was incubated for 20 min at 30 °C in the absence (lane CE) or presence of 1.8 μg of purified GST-Glc7 (B) or GST-Ppz11–344 (D) (lanes PP). One μg of this extract was also preincubated for 5 min at 30 °C with increasing amounts of purified Ypi1, Ypi1W53A, or GST proteins prior to the addition of the corresponding phosphatases. Reaction mixtures were then incubated for 20 min at 30 °C. Samples were analyzed by SDS-PAGE (%)) and immunodetected with anti-HA monoclonal antibody. Size standards are indicated in kDa. Shown are phosphorylated (P) and unphosphorylated (UP) forms of HA-Reg11–443.
Inhibitors

**FIG. 4.** PP2A phosphatase activity is not inhibited by Ypi1. p-Nitrophenyl phosphate dephosphorylation assays were carried out as described under “Materials and Methods.” 1.8 μg of GST-Glc7 (empty bars) or 30 ng of PP2A (solid bars) were incubated in the presence of p-nitrophenylphosphate (40 mM) as substrate. The indicated amounts of Ypi1 or okadaic acid were added prior to the incubation with the phosphatases. Values are means ± S.D. for at least three different assays, expressed as a percentage of phosphatase activity with respect to phosphatases. Values are means ± S.D. for at least three different assays, expressed as a percentage of phosphatase activity with respect to control without inhibitors.

until an absorbance at 660 nm of 0.6 was reached. Recovery from a factor arrest was performed as in Ref. 49. Budding index was monitored as in Ref. 49, and DNA content was determined by flow cytometry as in Ref. 50.

**RESULTS**

**Yfr003c Belongs to a Highly Conserved Family of Proteins Including a PP1 Protein Phosphatase Inhibitor—**YFR003c encodes a small protein (155 residues; 18 kDa, estimated molecular mass) very rich in hydrophilic residues (Asp + Glu content 19.4%); Ser + Thr content 14.8%; Lys + Arg content 16.8%) that shows an aberrant mobility in SDS-PAGE (it runs as a protein of around 30 kDa) and that is heat-stable (see below). All of these properties make this protein very similar to PP1 phosphatase inhibitors described in mammalian cells (16, 17). A protein BLAST analysis revealed that Yfr003c was highly homologous to a family of small proteins, one of which has been described as PP1 phosphatase inhibitor (Fig. 1). In fact, Yfr003c was previously postulated as the putative yeast homologue of mammalian PP1 inhibitor-3 (31). It is also important to notice that Yfr003c was strongly conserved throughout all eukaryotes, with homologues in yeast, insects, plants, worms, and mammals. Fig. 1A shows a phylogenetic tree of all of the Yfr003c homologues using the Genebee service (available on the World Wide Web at www.genebee.msu.su).

The Yfr003c Gene Product Interacts Physically with Glc7 and the PP1-related Phosphatase Ppz1—The yeast protein encoded by YFR003c (hereafter referred to YPI1, for yeast phosphatase inhibitor 1) was initially identified in a two-hybrid screening searching for Glc7-interacting proteins using LexA-Glc7 as bait (32). Yeast two-hybrid global analyses have also shown protein-protein interaction between Ypi1 and Glc7 (33, 34). To verify this interaction by an alternative experimental approach, we used an affinity pull-down assay system based on the expression of a GST-Glc7 fusion protein in bacteria. Purified GST-Glc7 was then used to bind HA-tagged-Ypi1 expressed in yeast cells. As shown in Fig. 2A (lane 2), HA-Ypi1 was detected in the fraction retained by GST-Glc7, corroborating the specific interaction between Glc7 and Ypi1. These results were in agreement with those presented recently on the interaction of Yfr003c (Ypi1) and Glc7 by affinity precipitation (51).

It has also been described that Ypi1 interacted in a two-hybrid analysis with the PP1-related phosphatase Ppz1 (37). To confirm this interaction, we performed a pull-down assay using a GST-Ppz1 C 344 fusion protein expressed in E. coli. This carboxyl-terminal domain of Ppz1 phosphatase expressed in bacteria has similar catalytic properties to the full-length protein (38). Yeast crude extracts expressing HA-Ypi1 were incubated with GST-Ppz1 C 344 bound to GSH-Sepharose and immunoblot analysis of the retained fraction indicated that Ypi1 was also able to interact physically with the Ppz1 phosphatase (Fig. 2B, lane 2).

Ypi1 Displays PP1 Phosphatase-inhibitory Activity—Since Ypi1 was able to bind both Glc7 and Ppz1 phosphatases, we investigated the possibility that Ypi1 might inhibit their phosphatase activity. Phosphatase activity was initially tested using p-nitrophenylphosphate as substrate. We incubated GST-Glc7 and GST-Ppz1 C 344 fusion proteins in the presence of different amounts of purified Ypi1 and determined the phosphatase activity of the mixture. As shown in Fig. 3, A and C, purified Ypi1 inhibited Glc7 phosphatase activity (up to 60% inhibition) in a dose-dependent manner. However, the addition of Ypi1 had a more modest effect on the Ppz1 phosphatase (up to 25% inhibition). On the contrary, the addition of Hal3, a specific inhibitor of Ppz1 phosphatase (38), did not affect Glc7 phosphatase activity but drastically reduced Ppz1 phosphatase activity (Fig. 3, A and C). The addition of GST alone did not alter the enzymatic activity of the corresponding phosphatases (Fig. 3, A and C). Since it has been described that some mammalian PP1 inhibitors are heat-stable (16, 17), we were interested in determining whether Ypi1 shares this characteristic. We found that Ypi1 was also heat-stable; treatment at 95 °C for 5 min did not abolish its inhibitory effect on Glc7 phosphatase activity (data not shown).

We extended our studies of the phosphatase inhibitory capacity of Ypi1 on Glc7 and Ppz1 phosphatase activities using Reg1 as a protein substrate. Reg1, a Glc7 regulatory subunit, is itself a physiological substrate of Glc7. Reg1 is phosphorylated by Snf1 protein kinase in response to a low glucose signal, and if glucose is added back to the medium, Reg1 undergoes dephosphorylation mediated by Glc7 (45). The phosphorylation status of Reg1 can be easily followed by changes in SDS-PAGE electrophoretic mobility when expressing an amino-terminal domain of the protein, Reg1 C 443 (Fig. 3B, lane CE) (45). We observed that after incubating the yeast extract expressing HA-Reg1 C 443 with purified GST-Glc7, the upper band, corresponding to the phosphorylated form of HA-Reg1 C 443, disappeared, indicating that Glc7 was able to dephosphorylate Reg1 protein in vitro (Fig. 3B, lane PP). The addition of increasing amounts of purified Ypi1 to the reaction mixture inhibited Glc7
phosphatase activity, confirming the inhibitory capacity of Ypi1. The addition of GST protein alone did not affect Glc7 phosphatase activity (Fig. 3B). We also tested whether purified GST-Ppz131–344 was able to dephosphorylate HA-Reg11–443. As shown in Fig. 3D, upon the addition of GST-Ppz131–344 to a yeast crude extract, the phosphorylated HA-Reg11–443 form disappeared, indicating that Ppz131–344 could also dephosphorylate HA-Reg11–443 in vitro (Fig. 3D, lane PP). However, the addition of increasing amounts of Ypi1 to the reaction mixture did not inhibit Ppz1 phosphatase activity significantly; we could observe only a very weak inhibition of the phosphatase activity at the highest concentration used (Fig. 3D).

To test whether Ypi1 was a specific inhibitor of PPI phosphatases, we used commercial PP2A phosphatase from bovine kidney (Calbiochem) and added different amounts of okadaic acid (a specific PP2A phosphatase inhibitor) or purified Ypi1 to the phosphatase reaction mixture, it did not affect PP2A phosphatase activities. Therefore, the presence of this conserved tryptophan residue was essential for both binding and inhibitory capacity on Glc7 and Ppz1 phosphatases.

Ypi1 Is Essential for Cell Viability—The systematic analysis of yeast deletion mutants indicates that a YFR003c null strain, in which the entire open reading frame has been deleted, is unviable (52). However, the YPI1 (YFR003c) open reading frame lies in the near vicinity of two well known essential genes, RPN11, which codes for a proteasome endopeptidase, and NIC96, which codes for a nucleoporin (53). The YPI1 coding sequence was on the Crick strand only 269 bp away from the RPN11 start codon (ATG) and 122 bp away from the NIC96 stop codon, both in the opposite strand. To confirm that lethality was strictly due to the elimination of YPI1, we disrupted the gene in a diploid strain and overexpressed wild-type Ypi1 protein in the heterozygous diploid mutant. Then we sporulated the diploid and performed tetrad analysis. In cells carrying an empty vector, we observed a 2:2 segregation of the lethal phenotype, and none of the viable spores contained the disrupted allele. On the other hand, the expression of Ypi1 from the plasmid allowed the growth of all four spores from a tetrad, two of them containing the ypi1Δ::KanMX4 disrupted allele (Fig. 5). These results indicated that the lack of Ypi1 was the direct cause for the lethality and suggested a relevant function for Ypi1 in yeast physiology.

Phenotypic Analyses of the Function of Ypi1 in Yeast—Since deletion of YPI1 was lethal, we studied the possible function of Ypi1 by overexpressing the protein. Since we have demonstrated that Ypi1 could function as a Glc7 phosphatase inhibitor, we tested whether some of the physiological processes regulated by Glc7 phosphatase were affected by the overexpression of Ypi1. First, we monitored growth rate in cells overexpressing Ypi1 and did not find significant differences with those carrying an empty plasmid. Then we investigated the role of Ypi1 in glycogen accumulation. It is known that in the absence of some specific regulatory subunits that recruit Glc7 phosphatase to the glycogen biosynthesis pathway (i.e. in gac1 mutants) or in glc7 partially defective mutants (deletion of GLC7 is lethal), the amount of cellular glycogen is low (54). We found that cells overexpressing Ypi1 contained lower levels of glycogen in comparison with cells carrying an empty plasmid (Fig. 6), suggesting an in vivo inhibition of Glc7 phosphatase activity by Ypi1.

Both Glc7 and Ppz1 phosphatases are known to play distinct roles in cell cycle regulation. We used the strain JC002, a sit4Δ deletion mutant in which the HAL3 gene is under the control of the tetO promoter and, therefore, cells can be blocked at the G1/S transition phase in the presence of doxycycline (55). As shown in Fig. 7A, when we tested whether high levels of Ypi1 could relieve such blockage, it could be observed that overexpression of Ypi1 allowed growth of strain JC002 under nonpermissive conditions, although much less vigorously than high copy expression of HAL3 from its own promoter. We also found that the ability to resume growth of the JC002 strain was lost when the W53A mutated version of Yip1p was tested (Fig. 7A).

Mutation of the SIT4 phosphatase gene is able by itself to produce a significant defect in growth under a number of different conditions. We show (Fig. 7B) that overexpression of Ypi1 improved growth of a sit4Δ strain at 37 °C, but it was ineffective to sustain vigorous growth in the presence of glycerol as the only carbon source. The growth defect of a sit4Δ mutant results from an expanded transition from G1 to S phase, which can be easily monitored after G1 blockage with the α-factor pheromone. As shown in Fig. 7, C and D, we determined budding index and DNA content of sit4Δ-arrested cells and demonstrated that high levels of Ypi1 accelerated recovery of sit4Δ cells from G1 blockage, albeit to a lesser extent.
than that achieved by high copy expression of HAL3. Expression of the W53A mutated version resulted in a phenotype equivalent to that of cells carrying an empty plasmid.

Both Glc7 and Ppz1 have been implicated in the maintenance of cell wall integrity, and cells defective for Glc7 or Ppz1 function aggravate the lytic phenotype of a slt2/mpk1Δ mitogen-activated protein kinase mutant. As shown in Fig. 8, overexpression of Ypi1 has a negative effect on growth of a slt2/mpk1Δ strain, although not as dramatic as high copy number expression of Hal3. This effect is particularly evident in synthetic medium, although it was also detectable in rich medium in the presence of relatively low concentrations of caffeine.

**DISCUSSION**

Type 1 protein phosphatase inhibitors represent a collection of modulators that maintain protein phosphatase activity under strict control. Some of these inhibitors are also regulated by different signaling pathways, representing alternative mechanisms for modulating phosphatase activity. For example, mammalian inhibitor-1 and DARPP-32 are converted to PP1 inhibitors only after phosphorylation driven by protein kinase A. In other cases, as in mammalian inhibitor-2, phosphorylation prevents the inhibitory capacity of the protein. Type 1 protein phosphatases are very well conserved throughout evolution. As in mammalian cells, the binding of specific regulatory subunits, which target the phosphatase to the corresponding substrates, modulates the action of yeast Glc7 protein phosphatase. However, until now, no Glc7 inhibitory proteins have been described in yeast. In silico analysis aimed to identify yeast homologues of mammalian inhibitor-1 or other related proteins has been unsuccessful, and the yeast homologue of mammalian inhibitor-2 (named Glc8) turned out to be an activator of Glc7 rather than an inhibitor (30). In this work, we describe the first endogenous Glc7 phosphatase inhibitor, which we named Ypi1. We show direct evidence that this protein, which possesses the VXW motif, physically interacts with Glc7 and inhibits its phosphatase activity in a manner that involves residue Trp53.

**Fig. 7.** Effect of overexpression of native and W53A mutated forms of Ypi1 on cell cycle. A, strain JC002 (sit4Δ tetO:HAL3) was transformed with the indicated plasmids and spotted on plates in the absence (−) or the presence of 20 μg/ml doxycycline (dox). Growth was monitored after 72 h at 30 °C. B, strain JA110 (sit4Δ) was transformed with plasmids, and growth was tested at 30 °C on rich medium with glucose (YPD) or glycerol (YPGly) as the carbon source and at 37 °C on YPD. Growth was documented after 120 h of incubation. C and D, strain JA100 (SIT4, circles) was transformed with empty plasmid pWS93, and strain JA110 (sit4Δ) was transformed with plasmids pWS93 (diamonds), pWS-Ypi1 (squares), or pHAL3 (triangles). Cells were grown to an A600 of 0.6 and arrested at G1 phase with α-factor as described under “Materials and Methods.” After removal of the pheromone, samples were taken at the indicated times and processed for determination of the percentage of budded cells by microscopic observation (C) or for monitoring DNA content by propidium iodine staining and flow cytometry (D).

**Fig. 8.** Overexpression of Ypi1 negatively affects growth of a slt2/mpk1Δ mitogen-activated protein kinase mutant. Strain JC10 (mpk1Δ) was transformed with the indicated plasmids, and growth was tested at 30 °C on SC medium lacking uracil and in rich medium (YPD) containing 1 M sorbitol or 2 mM caffeine. Growth was monitored in all cases after 60 h of incubation at 30 °C. Two dilutions of the cultures (1:5) are shown.
within this motif. We also show that mutation of this residue to alanine abolishes the phenotypic traits associated with overexpression of Ypi1, stressing the notion that this interaction is required for regulation of Glc7 function. It is interesting to notice that Ypi1 protein has the characteristic properties observed in mammalian PP1 inhibitors; it is heat-stable, small (155 amino acids), and very rich in hydrophilic residues. In addition, the Ypi1 sequence contains two putative protein kinase A phosphorylation sites, KKRT88 and KKRS111, and our preliminary results indicate that Ypi1 may be a phosphoprotein (data not shown), perhaps suggesting a possible role of this type of modification in regulating its inhibitory capacity.

Ypi1 is the yeast homologue of mammalian PP1 inhibitor-3 (31). Interestingly, when we performed a protein BLAST analysis on Ypi1, we found that Ypi1 and mammalian inhibitor-3 are members of a very well conserved family of proteins, all of them small in size, rich in hydrophilic residues, and harboring the characteristic PP1 binding motif VX(W/F). Members of this family are present in all eukaryotes, from yeast, insects, plants, and worms to mammals, suggesting that the function performed by yeast Ypi1 and mammalian inhibitor-3 is very well conserved throughout evolution. We confirm here that deletion of the YPI1 gene is lethal, suggesting a relevant function of this protein in the yeast physiology. Unfortunately, this fact prevents direct investigation of the function(s) of Ypi1 by simple deletion of the gene and evaluation of the associated phenotypes. Alternative approaches include the generation of conditional mutants or monitoring the phenotype of cells overexpressing the protein. Whereas the former approach is in progress in our laboratory, here we present evidence that cells overexpressing Ypi1 aggravate the lytic defect of a slt2Δmpk1 mutant in improving growth of the mutant in the presence of doxy-

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This phenotype seems to be specific to Glc7 inhibition, since glycolen levels in cells lacking Ppz1, Ppz2, or both phosphatases are equivalent to that of wild type cells (37, 62). In the same way, our assays (Fig. 3) indicate that Ypi1 substantially inhibits its Glc7 activity in vitro and has a lesser effect on Ppz1 activity.

All these results are compatible with a direct in vitro inhibitory role of Ypi1 on Glc7. A role as inhibitor of Ppz1, although possible, appears less likely under the conditions tested. However, it must be stressed that as Glc7 and Ppz1 catalytic subunits may interact with specific regulatory subunits in each biological process and this binding occurs in a mutually exclusive manner, overexpression of Ypi1 might interfere with the function of the catalytic subunit by displacing other regulators, regardless of whether Ypi1 itself has a physiological role in the pathway being assessed. A project being carried out in our laboratories, based on the characterization of phenotypes derived from the controlled loss of function of YPI1, will provide further insight into the physiological properties of this protein phosphatase inhibitor.

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