The Yeast Immunophilin Fpr3 Is a Physiological Substrate of the Tyrosine-specific Phosphoprotein Phosphatase Ptp1*

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The tyrosine-specific phosphoprotein phosphatase encoded by the Saccharomyces cerevisiae PTP1 gene dephosphorylates artificial substrates in vitro, but little is known about its functions and substrates in vivo. The presence of Ptp1 resulted in dephosphorylation of multiple tyrosine-phosphorylated proteins in yeast expressing a heterologous tyrosine-specific protein kinase, indicating that Ptp1 can dephosphorylate a broad range of substrates in vivo. Correspondingly, several proteins phosphorylated at tyrosine by endogenous protein kinases exhibited a marked increase in tyrosine phosphatase PTP1 in ptp1 mutant cells. One of these phosphotyrosyl proteins (p70) was also dephosphorylated in vitro when incubated with recombinant Ptp1. p70 was purified to homogeneity; analysis of four tryptic peptides revealed that p70 is identical to the recently described FPR3 gene product, a nuclearly localized proline rotamase of the FK506- and rapamycin-binding family. The identity of p70 with Fpr3 was confirmed in the demonstration that the abundance of tyrosine-phosphorylated p70 in ptp1 mutants was strictly correlated with the level of FPR3 expression; immobilized phosphotyrosyl Fpr3 was directly dephosphorylated by recombinant Ptp1. Site-directed mutagenesis demonstrated that the site of tyrosine phosphorylation is Tyr-184, which resides within the nucleolin-like amino-terminal domain of Fpr3. Protein kinase activities from yeast cell extracts can bind to and phosphorylate the immobilized amino-terminal domain of Fpr3 on serine, threonine, and tyrosine. Fpr3 represents the first phosphotyrosyl protein identified in S. cerevisiae that is not itself a protein kinase and is as yet the only known physiological substrate of Ptp1.

Phosphotyrosine-specific phosphoprotein phosphatases (PTPs)† have been identified in many evolutionarily divergent eukaryotes. These enzymes form a distinct superfamily and are unrelated in sequence to serine/threonine-specific phosphoprotein phosphatases (for reviews, see Refs. 1–3). All PTPs possess stretches of sequence similarity within their catalytic domains, including the active site consensus sequence (I/V)HCXAGXR(S/T)/G. This hallmark sequence contains an invariant Cys residue, which acts as the nucleophile during the dephosphorylation reaction, and a GXGXXG motif, which forms a phosphate-binding loop and is also found in nucleotide-binding proteins such as protein kinases and GTPases (4). The substrate-binding cleft of PTPs is surrounded by basic amino acids, which may explain the preference for acidic residues near the phosphorylated tyrosines in PTP substrates (5, 6).

In the budding yeast, Saccharomyces cerevisiae, dedicated tyrosine-specific protein kinases have not been identified. However, a number of genes encoding PTPs have been reported. These PTPs include both phosphotyrosine-specific and dual-specific enzymes as seen in higher eukaryotes. Two of the S. cerevisiae PTPs appear to be MAP kinase phosphatases. The dual-specific PTP encoded by the MSG5 gene dephosphorylates Fus3 and thereby contributes to the reversal of pheromone arrest (7). The PTP2 gene product is thought to dephosphorylate Hog1, a MAP kinase involved in osmoregulation (8). At least two S. cerevisiae PTPs are involved in cell cycle control: the CDC14 gene product is required for progression through S phase (9), and the product of the MIH1 gene, the S. cerevisiae homolog of the fission yeast cdc25, is thought to dephosphorylate the Cdc28 kinase (10). The YVH1 PTP gene is induced by nitrogen starvation and encodes a PTP that is required for maximal growth (11).

PTP1, the first PTP gene reported in budding yeast, was identified by the polymerase chain reaction using oligonucleotides corresponding to conserved PTP catalytic domain sequences as primers (12). Ptp1 appears to be phosphotyrosine specific and is comprised of a carboxyl-terminal catalytic domain and a unique 55-residue amino-terminal region of unknown function. Although Ptp1 is active in vitro against artificial substrates, the physiological role of Ptp1 is unknown; PTP1 disruption or overexpression does not overtly effect growth at extreme temperatures, sensitivity to different metal ions, osmotic stability, carbon source utilization, mating, or sporulation (12, 13). However, expression of PTP1 in fission yeast mimics cdc25 overexpression and leads to precocious mitosis (14). In addition, overexpression of PTP1 in S. cerevisiae rescues the synthetic lethality resulting from disruption of both PTP2 and PTC1, a gene encoding a putative Ser/Thr-specific phosphoprotein phosphatase of the PP2C class (15). These results suggest that when overproduced, Ptp1 may be capable of dephosphorylating Cdc2 and Hog1, but the relevance of these activities to normal Ptp1 function is unclear.

– 25185 –
Here, we describe the identification of yeast phosphotyrosyl proteins that are dephosphorylated by Ptp1 in vivo and present evidence that one Ptp1 substrate is the nucleolar immunophilin, Fpr3.

**MATERIALS AND METHODS**

**Yeast Strains and Culture Conditions**—Yeast strains used in this work are described in Table I. Yeast transformation was carried out using electroporation (16). To generate the strain YBB200, a 3.1-kilobase ClaI fragment containing a HIS3-disrupted ptp1 allele (13) (kindly provided by P. J. ames) was introduced into strain YPH499 (17) by DNA-mediated transformation. To generate strain YBB300, the same ClaI fragment was used to transform strain YBB100 (18). To generate the strain PJ55300, the pfrp2::His3 insertion mutation was introduced into strain PJ55-16C, following a procedure previously described (18). To generate YLW200, a 1.25-kilobase PvuII-Asel fragment containing a URA3-disrupted ptp1 allele was excised from plasmid pGEM-ptp1::URA3 (13) (kindly provided by R. Deschenes) and used to transform strain BJ2168. To induce transcription of genes driven by a GAL promoter, cultures were grown overnight to A600nm = 1 in defined medium containing 2% raffinose. To prepare large cultures, cells were grown for an additional 3 hr prior to harvesting. For large scale preparation of p70<sup>TM</sup>, strain PJ55-16A (ptp1::His3 ade2-1) was grown in YPD in a 200-liter fermenter with vigorous aeration to stationarystationary phase (A600nm = 3.5).

**Immunoblot Analysis**—For immunoblot analysis, cells were grown to late exponential phase (A600nm = 1). Cells were harvested by centrifugation at 4°C, 1000 g for 5 min, resuspended in 50 ml Tris-HCl (pH 7.2), 100 mM NaCl, 5 mM EDTA, and recentrifuged. Cell pellets were resuspended in an equal volume of ice-cold buffer A (50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 12 μM benzenesulfonyl fluoride, 5 μg phenylmethylsulfonyl fluoride, 100 μg Na<sub>3</sub>V<sub>0.5</sub>, and 0.5 μg/ml of each of the following: antipain, leupeptin, chymostatin, apronitin, and pepstatin.). The cells were lysed by vigorous agitation with glass beads as described (18). Lysates were clarified by centrifugation at 10,000 g for 10 min, brought to 1 x Laemmli sample buffer (19) by addition of an appropriate amount of a concentrated stock, and separated by SDS-PAGE. Alternatively, where indicated, lysates were prepared by an alkaline lysis method as described (20). Briefly, cell pellets were incubated at 4°C in 250 mM NaOH, 1% 2-mercaptoethanol for 10 min, and cellular proteins were precipitated in 8% trichloroacetic acid. Protein precipitates were rinsed twice in acetone, dried, and then resuspended prior to SDS-PAGE by boiling in Laemmli sample buffer.

Immunoblot analyses were carried out as described (21) with the following modifications. Cell proteins (100 μg of protein/ lane) were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF) (Immobilon P, Millipore). Blocking buffer containing 3% bovine serum albumin, 0.1% Tween 20, 0.5 mM NaCl, 0.5% Nonidet P-40, and 50 mM Tris-HCl (pH 7.5) was used for blocking, antibody incubation, and rinsing steps. The following antibodies were used at a concentration of 1 μg/ml to probe immunoblots: anti-phosphotyrosine monoclonal antibody (mAb) 4G10 (22) (Upstate Biotechnology), mAb F2B (23), mAb 699 (24), a polyclonal rabbit anti-phosphotyrosine antibody prepared by the method of Kamps and Sefton (25), rabbit anti-Fpr3 serum (18), anti-Src mAb 2-17 (Microbialogical Associates, Rockville, MD), and anti-Myc mAb 9E10 (26). Primary antibodies were detected by incubation with appropriate horseradish peroxidase-conjugated anti-immunoglobulin antibodies (Pierce), followed by chemilumi-
AffII and HindIII, and the AffII site was filled in with the Klenow fragment of DNA polymerase I. The resulting fragments were then ligated into the vector YEp351GAL (18), which had been opened with Salt, treated with Klenow, and then cut with HindIII. All constructs were verified by DNA sequencing.

Dephosphorylation of Phosphotyrosyl Proteins by GST-Ptp1 Fusion Proteins—To purify GST-Ptp1 fusion proteins, E. coli transformed with plasmids expressing the desired fusion were grown to $A_{600nm} = 1.0$, induced with 100 $\mu$M isopropl $\beta$-p-thiogalactoside, and harvested by centrifugation. Approximately 1 g of wet cells were resuspended in 5 ml of buffer B (buffer A devoid of vanadate) and broken by sonicated disruption. The lysates were centrifuged by centrifugation at 10,000 $\times g$ for 20 min, clarified by centrifugation at 12,000 $\times g$ for 30 min, and adjusted to a $A_{600nm}$ of 0.1 for protein A-Sepharose enrichment for 1 h. Then membranes were washed and resuspended in 30 ml of buffer C containing 20 mM NaCl, 5 mM EDTA, 100 $M$ dithiothreitol, 0.5 M $\mu$M Na$_3$VO$_4$, 20 mM glycerophosphate, 20 mM ATP, 0.1 $M$ MgCl$_2$, 0.1 $M$ EDTA, 0.5 M dithiothreitol, 0.5 M $\beta$-glycerophosphate, 0.1 $M$ Na$_3$VO$_4$, 2 $\mu$M leupeptin, 0.5 $M$ phenylmethylsulfonyl fluoride. An extract was prepared by vigorously agitating the resuspended pellet (approximately 50 $A_{600nm}$ units) with glass beads in 0.5 ml of extraction buffer for 5 min at 4°C. The resulting lysate was centrifuged at 12,000 $\times g$ for 10 min and adjusted in the protein concentration of 7 mg/ml by dilution with extraction buffer. A GST fusion encoding the amino-terminal 279 residues of Fpr3 (Fpr3N) was purified from bacterial extracts by adsorption to glutathione-Sepharose beads (18), and a sample (1 $\mu$L of beads containing 1 $\mu$g of GST-Fpr3N) was mixed with 200 $M$ [32P]-ATP (15 Ci/mmol). Reactions were carried out at 30°C for 3 h in a 0.4 in synthetic low phosphate medium (30), 0.4 in synthetic low phosphate medium containing 0.1 M Na$_3$VO$_4$, 0.4 in synthetic low phosphate medium containing 5 mM MgCl$_2$, and then resuspended to $A_{600nm} = 0.8$ in 5 ml of pretreated synthetic low phosphate medium containing 2 ml of 20% labeled PO$_4$ (DuPont NEN) (final specific activity, 8 Ci/mmol). After 3 h in a 30°C guratory water bath, the yeast were harvested by centrifugation, and cell proteins were prepared by alkaline lysis and trichloroacetic acid precipitation as described above. The dried precipitates were resuspended in boiling in 2% SDS for 5 min, centrifuged by centrifugation, diluted to 0.15% SDS with buffer A, and precleared by incubation with protein A-Sepharose for 1 h. Fpr3 was then immunoprecipitated with 0.4 $\mu$g of affinity-purified rabbit antibody raised against a bacterially expressed GST-Fpr3 fusion protein (18). The immunoprecipitates were resolved by SDS-PAGE, transferred to Immobilon P, and exposed to x-ray film. For phosphoamino acid analysis, the membranes were treated with Klenow, and then resuspended in 30 ml of buffer B containing 20 M NaCl, 5 mM EDTA, 100 $M$ dithiothreitol, 0.5 M $\beta$-glycerophosphate, 20 M $\mu$M Na$_3$VO$_4$, 2 $\mu$M leupeptin, 0.5 M phenylmethylsulfonyl fluoride. An extract was prepared by vigorously agitating the resuspended pellet (approximately 50 $A_{600nm}$ units) with glass beads in 0.5 ml of extraction buffer for 5 min at 4°C. The resulting lysate was centrifuged by centrifugation at 12,000 $\times g$ for 10 min and adjusted to a protein concentration of 7 mg/ml by dilution with extraction buffer. A GST fusion encoding the amino-terminal 279 residues of Fpr3 (Fpr3N) was purified from bacterial extracts by adsorption to glutathione-Sepharose beads (18), and a sample (1 $\mu$L of beads containing 1 $\mu$g of GST-Fpr3N) was mixed with $\sim 200 \mu$L of the lysate. Following incubation (4°C for 120 min), the remaining protein was removed by brief centrifugation and washed three times with 1 ml binding buffer (20 M Hepes (pH 7.8), 50 M NaCl, 2.5 M MgCl$_2$, 0.1 M EDTA, 0.05% Triton X-100). The washed beads were resuspended in 30 $M$ of kinase reaction buffer (20 M Hepes (pH 7.8), 20 M MgCl$_2$, 20 M $\beta$-glycerophosphate, 20 M $\mu$M p-nitrophenylphosphate, 0.1 M Na$_3$VO$_4$), containing 20 $\mu$L of 32P-ATP (15 Ci/mmol). Reactions were carried out for 30 min at 30°C.

RESULTS

Ptp1 Has Broad Substrate Specificity—The product of the PTP1 gene (Ptp1) dephosphorylates several artificial substrates in vitro (12), suggesting that it maybe capable of dephosphorylating a broad range of substrates in vivo. To explore this possibility, we compared the levels of tyrosine phosphorylation of yeast proteins in strain P[55–16C], which harbors a disruption of the PTP1 gene (ptp1Δ), and the congenic PTP1 strain P[55–16A]. To enhance total cellular tyrosine phosphorylation, these strains were transformed with a multi-copy plasmid expressing a known protein-tyrosine kinase, p60$^{v-src}$, under the control of the GAL1 promoter (28, 33). Lysates prepared from these strains were examined by immuno-blotting with anti-Src and anti-phosphotyrosine antibodies. The level of v-src expression in ptp1Δ cells was similar to that of the control PTP1 cells (Fig. 1, bottom panel). In PTP1 strains,
proteins phosphorylated by p60v-src greatly elevated (Fig. 1, photyrosyl proteins and their extent of phosphorylation were 28 and 33). In the absence of Ptp1, both the number of phosphorylated p60v-src and at least 10 different cellular proteins (Fig. 1, lanes 1, 2–17 (lower panel, lanes 1, 2); Molecular mass (in kDa) markers are indicated on the left.

p60v-src expression resulted in tyrosine phosphorylation of at least 10 different cellular proteins (Fig. 1, lane 1; see also Refs. 28 and 33). In the absence of Ptp1, both the number of photyrosyl proteins and their extent of phosphorylation were greatly elevated (Fig. 1, lane 2). This result suggests that Ptp1 is able to dephosphorylate many different photyrosyl proteins and has a broad substrate specificity in vivo. In contrast, disruption of PTP2 and MIH1 did not affect the level of tyrosine phosphorylation induced by v-src (data not shown).

To determine whether Ptp1 can dephosphorylate in vitro the proteins phosphorylated by p60v-src, a bacterially expressed GST-Ptp1 fusion protein was incubated with the photyrosyl proteins in lysates from either PTP1 or ptp1Δ cells expressing v-src. The level of tyrosine phosphorylation was drastically reduced by incubation with GST-Ptp1 (Fig. 1, lanes 3 and 4). In contrast, incubation with catalytically inactive GST-Ptp1(C252A) did not significantly reduce the level of tyrosine phosphorylation (Fig. 1, lanes 5 and 6). This result confirms that Ptp1 has broad substrate specificity in vitro.

Disruption of PTP1 Enhances Detection of Endogenous Photyrosyl Proteins—Physiological substrates of S. cerevisiae Ptp1 have not been reported. The phosphorylated proteins detected in ptp1Δ yeast expressing v-src may not represent physiological substrates of Ptp1, as there is no evidence that these proteins are phosphorylated in the absence of v-src expression.

However, the experiment described above suggested that disruption of PTP1 might lead to detectable increases in the level of photyrosine in authentic Ptp1 substrates that are phosphorylated by endogenous kinases in vivo. We therefore sought to detect photyrosyl proteins in Ptp1-deficient yeast that were not expressing any exogenous tyrosine kinase. Extracts of a ptp1Δ ptp2Δ mih1Δ triple mutant and its congenic wild-type strain were examined by immunoblotting with several different anti-phosphotyrosine antibodies (Fig. 2). Many of the bands were common to the wild-type and triple mutant strains and appeared to correspond to abundant proteins because they were congruent with species visualized by staining with Coomassie Blue (data not shown). Reaction of these proteins with the anti-phosphotyrosine antibodies was variable and likely to be nonspecific because it was not reversed by competition with 50 \mu M phosphorysine (data not shown). In contrast, several proteins reactive with anti-phosphotyrosine antibody were detected in the triple PTP mutant that were not detectable in the wild-type strain (Fig. 2). Proteins of apparent molecular masses of 175 and 116 kDa were recognized by mAb FB2 (lanes 3 and 4), a protein of apparent molecular mass of 170 kDa was recognized by the rabbit polyclonal antibody (lane 7), and a protein of apparent molecular mass of 70 kDa was recognized by all of the antibodies with the exception of mAb 6G9 (lanes 1, 3, and 7). The latter protein, designated p70, was chosen for further study.

To determine whether the activity of other S. cerevisiae PTPs affected the tyrosine phosphorylation state of p70 (or any other protein) during normal growth, lysates of strains containing disruptions in PTP1, PTP2, and MIH1 were compared by anti-phosphotyrosine antibody immunoblotting. Anti-phosphotyrosine antibody recognized p70 only in strains disrupted for PTP1 (Fig. 3A). Disruption of PTP2 or MIH1 did not result in a detectable increase in the level of tyrosine phosphorylation on p70 or in the appearance of additional photyrosyl proteins (Fig. 3A, lanes 3 and 4). These results suggest that photyrosyl p70 is dephosphorylated only by Ptp1. In addition, these results support the conclusion that Ptp2, which is thought to dephosphorylate the Hog1 kinase (8), and Mih1, which is thought to dephosphorylate the Cdc28 kinase (10), have more restricted substrate specificities than Ptp1.

Ptp1 Dephosphorylates p70 in Vivo and In Vitro—The find-
shown). These results suggest that p70 is a direct substrate of Ptp1 but not by GST-Ptp1(C252A) (Fig. 4, lane 3) were grown in the presence of galactose and lysed by agitation with glass beads. Lysates were analyzed by immunoblotting with anti-phosphotyrosine mAb 4G10. Panel B, restoration of PTP1 expression suppresses tyrosine phosphorylation of p70. Lysates of the wild-type strain PJ58–8B (lane 1), the ptp1Δ strain PJ58–2B (lanes 2–4), or p70-enriched S-Sepharose fractions from strain PJ58–2B (lanes 5–7) were incubated with buffer (lanes 1, 2, 5), GST-Ptp1 (lanes 3, 6), or GST-Ptp1(C252A) (lanes 4, 7) and then boiled in SDS sample buffer and analyzed by immunoblotting with anti-phosphotyrosine mAb 4G10. The 65-kDa GST-Ptp1 fusion proteins (lanes 3–7) are stained nonspecifically by the 4G10 antibody.

Ptp1 rather than a substrate of another yeast phosphatase activated by Ptp1.

Tryptic peptides were generated from purified p70, and five were sequenced. Four of the five sequences matched precisely the amino acid sequence of S. cerevisiae Fpr3, a recently identified nuclear FK506-binding protein (Fig. 6) (18, 34, 35). FK506-binding proteins (FKBPs) are immunophilins that bind the structurally related immunosuppressive drugs, FK506 and rapamycin. The formation of complexes between these drugs and the predominant cytosolic FKBP, FKBP-12, inhibits signal transduction pathways in both vertebrates and yeast, but the normal functions of these proteins are unknown (see "Discussion"). Genetic analysis confirmed that p70 is identical to Fpr3. When a ptp1Δ strain was transformed with a high copy plasmid expressing galactose-inducible FPR3, p70 was greatly overproduced; conversely, p70 was completely absent in the ptp1Δ fpr3Δ strain (Fig. 7A). These findings, together with the sequence of the tryptic peptides derived from p70, verify that p70 is Fpr3. The anomalous electrophoretic mobility of Fpr3 (calculated molecular mass, 47 kDa) has been noted previously (18).

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from bacteria (which lack PTPs), we conclude that Ptp1 can directly dephosphorylate Fpr3. Some phosphotyrosyl-Fpr3 remained phosphorylated following Ptp1 treatment; this may be because the phosphotyrosine residue was inaccessible in a fraction of the immobilized molecules.

Identification of the Tyrosine Phosphorylation Site in Fpr3—Fpr3 contains an amino-terminal nucleolin-like segment (amino acids 20–290) and a COOH-terminal immunophilin domain (amino acids 291–413) (18). To determine which of these two regions of Fpr3 is subject to tyrosine phosphorylation, each region was expressed independently in fpr3Δ and ptp1Δ fpr3Δ yeast strains. In the ptp1Δ fpr3Δ strain, phosphotyrosine was readily detected in both full-length Fpr3 and its amino-terminal domain (Fig. 8A, lanes 2 and 4), but phosphotyrosine was not detected in the COOH-terminal domain (Fig. 8A, lane 6). These results indicate that the site of tyrosine phosphorylation in Fpr3 is one or more of the seven tyrosine residues within the nucleolin-like domain (Fig. 6).

Inspection of the sequence of the amino-terminal domain of Fpr3 revealed that two of the Tyr residues (Tyr-184 and Tyr-189) are immediately preceded by two or more acidic residues (Fig. 6). This sequence context is favored by many of the Tyr-specific protein kinases in higher eukaryotes (36). To determine if Tyr-184 and -189 were sites of tyrosine phosphorylation, these residues were altered by site-directed mutagenesis of FPR3. Neither the Y184F,Y189F double mutant nor the Y184F single mutant contained detectable phosphotyrosine (Fig. 8B, lanes 2 and 3). In contrast, the Y189F mutant possessed just as high a level of phosphotyrosine as wild-type Fpr3 (Fig. 8B, lanes 1 and 4). Thus, Tyr-184 appears to be the sole site of tyrosine phosphorylation of Fpr3.

Fpr3 Is Phosphorylated on Ser, Thr, and Tyr—To assess the phosphorylation state of Fpr3 in vivo, Fpr3 was immunoprecipitated from [32P]orthophosphate-labeled cells and subjected to phosphoamino acid analysis (Fig. 9, panels 1 and 2). The relative phosphoamino acid content of Fpr3 from ptp1Δ yeast
Panel A,

was ~85% phosphoserine, ~11% phosphothreonine, and ~4% phosphotyrosine (values represent the mean of three independent experiments with S.E. of <1%). No phosphotyrosine was detectable in Fpr3 immunoprecipitated from PTP1 yeast. When the amount of radiolabel in Fpr3 was normalized to the amount of FPR3 protein quantitated by staining, the stoichiometry of phosphate incorporated per mole of Fpr3 was ~3 mol of phosphoserine, 0.4 mol of phosphothreonine, and in the ptp1Δ strain, 0.2 mol of phosphotyrosine.

A Protein-tyrosine Kinase Binds to and Phosphorylates Fpr3 in Vitro—To begin to characterize the protein kinases(s) responsible for phosphorylating Fpr3, a bacterially produced fusion protein consisting of GST and the amino-terminal 279 residues of Fpr3 (GST-Fpr3N) was adsorbed onto glutathione-Sepharose beads, washed, and incubated with yeast lysate. The beads were then washed exhaustively and incubated in a protein kinase reaction buffer containing [γ-32P]ATP. Radiolabel was incorporated into GST-Fpr3N but not onto GST alone, suggesting that a protein kinase had bound to and phosphorylated the amino-terminal domain of the immobilized Fpr3 (data not shown). Phosphorylated GST-Fpr3N was subjected to phosphoamino acid analysis and was found to contain phosphoserine, phosphothreonine, and phosphotyrosine (Fig. 9, panel 3), indicating that one or more protein kinases capable of associating with and phosphorylating the amino-terminal domain of Fpr3 are present in yeast cell extracts. In addition, incubation of the phosphorylated GST-Fpr3N with Ptp1 prior to phosphoamino acid analysis led to the complete removal of phosphotyrosine but no significant change in levels of phosphoserine or phosphothreonine (data not shown), supporting previous findings (12) that Ptp1 is tyrosine specific.

DISCUSSION

The Substrate Specificity of Ptp1—We have shown here that in yeast cells lacking Ptp1, the nucleolar immunophilin Fpr3 and several unidentified proteins exhibit enhanced levels of tyrosine phosphorylation. In the case of Fpr3, we demonstrated that this protein is a direct substrate of Ptp1 in vitro. We also found that Ptp1 can substantially reverse phosphorylation of a
large number of yeast proteins phosphorylated at tyrosine by \( p_60^{\text{src}} \). These findings indicate that Ptp1 is a broad specificity PTP, similar to the mammalian enzyme PTP1B (37), which, when expressed in yeast (28), is also capable of dephosphorylating numerous proteins phosphorylated by \( p_60^{\text{src}} \). Additionally, when overexpressed in Schizosaccharomyces pombe, either \( S.\text{cerevisiae} \) PTP1 or mammalian PTP1B can complement a mutation in an endogenous PTP, Cdc25, and activate the cell cycle regulator, Cdc2 (14). In contrast to Ptp1, Ptp2 recognizes a very limited number of substrates. Disruption of \( S.\text{cerevisiae} \) PTP2 either in the presence or absence of \( v^\text{src} \) expression did not cause detectable increase in protein phosphotyrosine. These results are consistent with the previous observation that Ptp2 is unable to dephosphorylate artificial substrates in vitro (13).

The broad substrate specificity of Ptp1 suggests several possible functions for the enzyme. One extreme possibility is that Ptp1 may totally lack specificity for protein substrates, and function simply to reverse adventitious tyrosine phosphorylation by error-prone or promiscuous Tyr-specific or dual-specific protein kinases. Consistent with this idea, we observed that ptp1\( \Delta \) yeast were killed by mutants of the v-src tyrosine kinase that were only partially growth inhibitory in PTP1 strains. However, other observations suggest that Ptp1 has some level of substrate specificity and thus that it may have a more specific role in yeast cell physiology. It is clear that Ptp1 is unable to fulfill the functional niches occupied by other PTPs in \( S.\text{cerevisiae} \). The fact that cells carrying mutations in a PTP-encoding gene, CDC14, undergo a cell cycle arrest (9) is evidence that, under normal conditions, Ptp1 cannot dephosphorylate the substrate(s) of Cdc14. Recent genetic evidence indicates that Ptp2 may function by dephosphorylating Hog1, the terminal MAP kinase of the osmosensory signaling pathway. The Sln1 gene encodes a histidine-protein kinase receptor that mediates this osmosensory pathway. Overexpression of PTP2 (but not of PTP1) rescues sln1 mutants, and normal expression of PTP2 (but not of PTP1) can compensate for a mutation in the functionally related phosphatase, Ptc1 (8, 15).

It will be of interest to determine whether the limitations on the activity of Ptp1 are a result of its subcellular localization (see below) or an inability to recognize and dephosphorylate certain phosphotyrosyl proteins.

Ptp3 Phosphorylation—The findings presented here indicate that the yeast nuclear phosphotyrosyl protein Fpr3 is a substrate of Ptp1 in vivo. Phosphotyrosyl Fpr3 was detected in all of the ptp1\( \Delta \) strains and in none of the PTP1 strains deficient in Ptp2 or Mih1, indicating that Fpr3 is a physiological substrate of Ptp1 but not of other yeast PTPs.

At present, we do not know whether tyrosine phosphorylation affects Fpr3 function. Indeed, the precise cellular function of Fpr3 is unknown, but the properties of related mammalian and yeast immunophilins provide several clues. The peptidyl-prolyl isomerase activity of immunophilins suggests that they may catalyze protein folding (reviewed in Ref. 38). The immunosuppressant drugs, FK506 and rapamycin, which mimic the peptidyl-prolyl bond, bind to the FKBP class of immunophilins. In mammalian T-cells, the complex of drug and immunophilin blocks signal transduction. In yeast, exposure to FK506 inhibits calcineurin-mediated signal transduction and certain amino acid permeases (39), while exposure to rapamycin is lethal (40). Three FKBFs have been described in \( S.\text{cerevisiae} \) Fpr1, Fpr2, and Fpr3. Fpr1, a homolog of the mammalian FKBP12, is a cytosolic protein with high affinity for FK506 and rapamycin. FPR1-deficient yeast are resistant to these drugs, indicating that Fpr1 is largely responsible for mediating drug toxicity (41–43). \( S.\text{cerevisiae} \) FPR2, a homolog of mammalian FKBP13, may be involved in the proper folding of proteins in the ER (44, 45).

Fpr3 is an abundant nuclear protein that is dispensable for growth. Yeast with disruptions in FPR3, including fpr1 fpr2 fpr3 strains, grow normally under a variety of growth conditions. The drug-binding and proline isomerase activities of Fpr3 are mediated by the conserved immunophilin domain, which represents the carboxyl-terminal third of Fpr3. When this domain is expressed independently, it is retained in the cytoplasm and restores FK506 and rapamycin sensitivity in fpr1\( \Delta \) strains. The amino-terminal two-thirds of Fpr3 contains regions of acidic and basic residues and is responsible for localization of Fpr3 in the nucleus (18, 34, 35). This portion of Fpr3 exhibits some sequence similarity to nucleolin, a major nuclear protein thought to be involved in ribosome assembly and shuttling of RNA or proteins through nuclear pores (for review, see Ref. 46).

Our findings also indicate that Fpr3 is tyrosine phosphorylated within the amino-terminal nuclear localization domain. Preliminary immunofluorescence studies suggest that Ptp1 is localized primarily in the cytosol.3 These observations raise the possibility that Fpr3 might be dephosphorylated by Ptp1 prior to its entry into the nucleus and that tyrosine phosphorylation and dephosphorylation of Fpr3 might regulate its subcellular localization. By dephosphorylating Fpr3, Ptp1 might also play a role in regulating the catalytic activity of Fpr3 and/or the ability of Fpr3 to associate with other proteins in the nucleus. The Y184F mutant of Fpr3 does not undergo tyrosine phosphorylation and will serve as a useful reagent to study the possible effects of tyrosine phosphorylation/dephosphorylation on Fpr3 localization and catalytic activity.

The Fpr3 Tyrosine Kinase—Our results indicate that Fpr3 is only transiently tyrosine phosphorylated under normal growth conditions, since phosphotyrosyl-Fpr3 does not accumulate appreciably in PTP1 strains. Other \( S.\text{cerevisiae} \) proteins reported to contain phosphotyrosine include Cdc28 kinase, which is thought to be phosphorylated by Swe1, a \( S.\text{cerevisiae} \) homolog of S. pombe Wee1 (47), MAP kinases, which are phosphorylated by MAP kinase kinases (MEKs) (48, 49), and dual-specific

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3 J. Trager and G. S. Martin, unpublished results.

4 L. Wilson and G. S. Martin, unpublished results.
food kinases such as Mck1 (50), Spk1 (51), and casein kinase I (52) that autophosphorylate on tyrosine. Thus, Fpr3 is the first tyrosine-phosphorylated protein identified in yeast that is not itself a protein kinase.

Because dedicated tyrosine-specific protein kinases have not been identified in unicellular eukaryotes, it is possible that a dual specificity kinase is responsible for the phosphorylation of Fpr3 at Tyr-184. Alternatively, the Fpr3 tyrosine kinase might be a novel yeast kinase and is tyrosine-specific. We have shown here that yeast extracts contain an activity or activities that will be able to construct a yeast strain with a phosphotyrosine phosphatase-deficient strain Pj58–2B, Pj58–8B, Pj55–16A, Pj55–16C, and Pj55–16D, plasmid YEp51-PTP1, and the ptp1::HIS3 knock-out construct, to R. Deschenes for providing pGEM-tpl1::URA3, to S. Kanter for providing mAb 6G9, and to D. Brazil, M.-Y. Lim, and L. England for helpful discussions. H. Saito and his collaborators (personal communication) have also detected tyrosine phosphorylation of a 70-kDa protein and at least 10 additional proteins in ptp1Δ yeast.

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