The Yeast Phosphotyrosyl Phosphatase Activator Is Part of the Tap42–Phosphatase Complexes

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Phosphotyrosyl phosphatase activator PTPA is a type 2A phosphatase regulatory protein that possesses an ability to stimulate the phosphotyrosyl phosphatase activity of PP2A in vitro. In yeast *Saccharomyces cerevisiae*, PTPA is encoded by two related genes, *RRD1* and *RRD2*, whose products are 38 and 37% identical, respectively, to the mammalian PTPA. Inactivation of either gene renders yeast cells rapamycin resistant. In this study, we investigate the mechanism underlying rapamycin resistance associated with inactivation of PTPA in yeast. We show that the yeast PTPA is an integral part of the Tap42–phosphatase complexes that act downstream of the Tor proteins, the target of rapamycin. We demonstrate a specific interaction of Rrd1 with the Tap42–Sit4 complex and that of Rrd2 with the Tap42–PP2Ac complex. A small portion of PTPA also is found to be associated with the AC dimeric core of PP2A, but the amount is significantly less than that associated with the Tap42-containing complexes. In addition, our results show that the association of PTPA with Tap42–phosphatase complexes is rapamycin sensitive, and importantly, that rapamycin treatment results in release of the PTPA–phosphatase dimer as a functional phosphatase unit.

INTRODUCTION

Protein phosphatase 2A (PP2A) represents a major group of serine/threonine phosphatases that is highly conserved and ubiquitously expressed in eukaryotes (Mumby and Walter, 1993). PP2A has been implicated in a wide spectrum of cellular processes, including signal transduction, metabolism, cell cycle progression, gene expression, and protein translation (Schonthal, 1998; Virshup, 1999; Sontag, 2001; Zabrocki *et al.*, 2002). The core enzyme of PP2A comprises a catalytic subunit, the C-subunit (PP2Ac), and a scaffolding protein, the A subunit. The substrate specificity of PP2A as well as its intracellular localization are defined by an array of distinct groups of B-type regulatory subunits, termed B, B', and B'', that bind to the AC dimeric core to form a variety of heterotrimeric complexes (Mumby and Walter, 1993). In yeast *Saccharomyces cerevisiae* two closely related genes, *PPH21* and *PPH22*, encode two functionally redundant catalytic subunits of PP2A (PP2Ac) (Sneddon *et al.*, 1990), which exist primarily in cells as a heterotrimeric complex with the A and B subunits (Stark, 1996). The product of the *TPD3* gene serves as the A subunit (van Zyl *et al.*, 1992), and two distinct proteins, encoded by *CDC55* and *RTS1*, serve as alternative B subunits (Healy *et al.*, 1991; Shu *et al.*, 1997).

In addition to the A and B subunits, PP2Ac has been found to associate with a novel regulatory subunit, termed Tap42 in yeast, and α -4 protein/mTap42 in mammalian cells (Di Como and Arndt, 1996; Murata *et al.*, 1997; Prickett and Brautigan, 2004). The Tap42–PP2Ac complex is formed independently of the A and B subunits and thus represents a unique form of the phosphatase (Jiang and Broach, 1999). However, the complex in yeast seems to be a minor form of

the phosphatase in comparison with the PP2A holoenzyme, because only a small fraction (~5%) of PP2Ac is found to be associated with Tap42 under normal growth conditions (Di Como and Arndt, 1996). Homologues of Tap42 have been found in various organisms, and in all the cases examined to date, have been found to associate with PP2Ac or 2A-like phosphatases (Murata *et al.*, 1997; Chen *et al.*, 1998; Harris *et al.*, 1999), suggesting that the Tap42–PP2Ac complex is conserved during evolution.

It has been shown that Tap42 associates with PP2Ac only in cells actively growing but not in those entering stationary phase. The dynamic interaction between Tap42 and PP2Ac is regulated by the Tor proteins, and is sensitive to rapamycin, a macrolide antibiotic that specifically inhibits Tor function (Di Como and Arndt, 1996). In yeast, the Tor proteins are the key components of a nutrient sensing signaling cascade that controls a wide spectrum of growth-related events in response to nutrient availability and conditions (Schmelzle and Hall, 2000). Tap42 has been found to be phosphorylated both in vivo and in vitro by the Tor proteins, and Tor-dependent phosphorylation is believed to promote its interaction with phosphatases (Jiang and Broach, 1999). On the other hand, mutations in *TAP42* have been found to confer yeast cell rapamycin resistance, suggesting that Tap42 is a key effector of the Tor-mediated signaling (Di Como and Arndt, 1996). In addition to PP2Ac, Tap42 has been found to associate with many 2A-like phosphatases in yeast, including Sit4, a phosphatase that plays a major role in Tor-mediated gene expression (Beck and Hall, 1999; Jacinto *et al.*, 2001). As in the case with PP2Ac, the interaction of Tap42 with Sit4 is also rapamycin sensitive (Di Como and Arndt, 1996).

Although PP2A displays mainly Ser/Thr phosphatase activity, in vitro studies have demonstrated an intrinsic phosphotyrosyl phosphatase activity, which can be significantly stimulated by a cellular factor, called phosphotyrosyl phosphatase activator (PTPA) (Cayla *et al.*, 1990; Janssens *et al.*, 1998). Despite this in vitro finding, the in vivo function of

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Table 1. Strains used in this study. All strains are derivatives of W303 (*ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 GAL*) except those indicated (Thomas and Rothstein, 1989)

Strain	Genotype ^a	Source
Y062 ^b	<i>MAT α ura3 leu2 his3 trp1 SSD1-v1</i>	Laboratory stock
Y162	<i>MAT α (HA)₃PPH21</i>	Jiang and Broach (1999)
Y275 ^b	<i>MAT α gln3::KanMX4 ura3 leu2 trp1 met15</i>	Laboratory stock
Y397 ^b	<i>MAT α sit4::HIS3 SSD1-v1 ura3 leu2 his3 trp1</i>	Laboratory stock
Y531	<i>MAT α pph22::HIS3, pph21::LEU2</i>	Laboratory stock
Y661	<i>MAT α W.T. (W303-1A)</i>	Laboratory stock
Y663	<i>MAT α/α W.T.</i>	Laboratory stock
Y841	<i>MAT α rrd1::HIS3</i>	This study
Y842	<i>MAT α rrd1::HIS3</i>	This study
Y843	<i>MAT α rrd2::HIS3</i>	This study
Y844	<i>MAT α rrd2::HIS3</i>	This study
Y846	<i>MAT α rrd1::HIS3 [pRS314-RRD1(myc)₁₃]</i>	This study
Y847	<i>MAT α rrd1::HIS3 [pRS314]</i>	This study
Y848	<i>MAT α rrd2::HIS3 [pRS314-RRD2(myc)₁₃]</i>	This study
Y850	<i>MAT α rrd1::HIS3 rrd2::HIS3 [pRS314-RRD1(myc)₁₃]</i>	This study
Y852	<i>MAT α rrd1::HIS3 rrd2::HIS3 [pRS314-RRD2(myc)₁₃]</i>	This study
Y869	<i>MAT α rrd1::HIS3 rrd2::HIS3 [pRS416-RRD1]</i>	This study
Y871 ^a	<i>MAT α rrd1::KanMX4 rrd2::HIS3 his3 leu2 met15 ura3</i>	Fellner <i>et al.</i> (2003)
Y874	<i>MAT α rrd1::HIS3 rrd2::HIS3 [pRS415-SSD1-v1]</i>	This study
Y884 ^b	<i>MAT α rrd1::KanMX4 leu2 ura3 his3 met15</i>	This study
Y885 ^b	<i>MAT α rrd2::HIS3 leu2 ura3 his3</i>	This study
Y886 ^b	<i>MAT α rrd1::KanMX4 sit4::HIS3 leu2 ura3 his3 trp1</i>	This study
Y887 ^b	<i>MAT α rrd2::HIS3 sit4::HIS3 leu2 ura3 his3 trp1</i>	This study
Y929	<i>MAT α rrd1::HIS3 rrd2::HIS3 cdc55::(HA)₃CDC55-URA3 [pRS314-RRD1(myc)₁₃]</i>	This study
Y930	<i>MAT α rrd1::HIS3 rrd2::HIS3 cdc55::(HA)₃CDC55-URA3 [pRS314-RRD2(myc)₁₃]</i>	This study
Y964 ^b	<i>MAT α sit4::HIS3 gln3::KanMX4 SSD1-v1 ura3 leu2 met15</i>	This study

^a Plasmids are indicated in square brackets.^b Derivatives of S288C.

PTPA remains unclear. Like other PP2A regulatory proteins, PTPA is highly conserved from yeast to human. In yeast, *RRD1* and *RRD2* encode two yeast homologues of PTPA that are 38 and 37% identical, respectively, to the mammalian counterpart (Rempola *et al.*, 2000). Inactivation of either gene has no major effect on cell growth. However, inactivation of both genes drastically impairs cell growth and is lethal in some strain backgrounds, suggesting that PTPA activity is essential for cell growth (Rempola *et al.*, 2000; Van Hoof *et al.*, 2000; Mitchell and Sprague, 2001).

Despite the relatively mild growth defect associated with the *rrd1* and *rrd2* deletions, cells lacking either gene display various mutant phenotypes, among which is resistance to rapamycin (Rempola *et al.*, 2000). The drug-resistant trait is indicative of a role for the yeast PTPA in the Tor-mediated signaling pathway. Because the phosphatases involved in the pathway are the Tap42-containing complexes, including

both the Tap42–PP2Ac and Tap42–Sit4 complexes, the rapamycin-resistant trait associated with the *rrd1* and *rrd2* mutants raises the possibility that the Rrd proteins may regulate these complexes. In the present study, we tested this possibility by examining the potential interactions between the Rrd proteins and the Tap42–phosphatase complexes.

MATERIALS AND METHODS

Yeast Strains and Reagents

Strains used in this study are listed in Table 1. Yeast cells were normally grown in YP or synthetic complete (SC) medium lacking appropriate amino acid(s) for selection. All media contained 2% glucose as a carbon source. Standard methods were used for yeast transformation and other manipulations (Guthrie and Fink, 1991). Table 2 lists all the plasmids used in this study. Rapamycin (Sigma-Aldrich, St. Louis, MO) was stored in 10% Tween 20 and 90% ethanol at a concentration of 1 mg/ml and was added to growth medium to a final concentration of 200 ng/ml (liquid) or 100 ng/ml (plates). Anti-

Table 2. Plasmids used in this study

Plasmid	Gene	Vector	Source
pRS314		<i>CEN TRP1</i>	Sikorski and Hieter (1989)
pRS416-RRD1	<i>RRD1</i>	<i>CEN URA3</i>	This study
pRS416-GLN3(myc) ₁₃	<i>GLN3(myc)₁₃</i>	<i>CEN URA3</i>	Wang <i>et al.</i> (2003)
pRS314-RRD1(myc) ₁₃	<i>RRD1(myc)₁₃</i>	<i>CEN TRP1</i>	This study
pRS314-RRD2(myc) ₁₃	<i>RRD2(myc)₁₃</i>	<i>CEN TRP1</i>	This study
pRS406-cdc55(HA) ₃	<i>cdc55(HA)₃</i>	<i>URA3</i>	This study
pRS415-SSD1-v1	<i>SSD1-v1</i>	<i>CEN LEU2</i>	Laboratory stock
pRS416-ADHp-GST	<i>GST</i>	<i>CEN URA3</i>	Wang <i>et al.</i> (2003)
pRS416-ADHp-GST-SIT4	<i>GST-SIT4</i>	<i>CEN URA3</i>	Wang <i>et al.</i> (2003)

Tap42, Sit4, and Tpd3 antibodies have been described previously (Jiang and Broach, 1999). Anti-myc (9E10) and anti-HA (12CA5) antibodies were purchased from Roche Diagnostics (Indianapolis, IN) and used according to manufacturer's instructions. Protein A beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid and Strain Construction

Cloning and Epitope Tagging of RRD1 and RRD2. The *RRD1* gene was amplified from yeast genomic DNA by PCR by using high-fidelity *Taq* polymerase (Roche Diagnostics). The 5' primer for the PCR was placed ~480 bp upstream of the initial codon of the gene, and the 3' primer was placed immediately before the stop codon. The PCR product was digested with *KpnI* and *BamHI* whose sites were incorporated into the product via the two primers and cloned into pRS314 (*CEN TRP1*) at the corresponding sites. The resulting plasmid was then digested with *BamHI* and ligated with a 0.8-kb *BamHI*-*BglIII* DNA fragment containing a sequence for 13 tandem copies of myc epitope followed by an *ADHI* termination sequence that was excised from pFA6a-13myc-*TRP1* (Longtine *et al.*, 1998). The *BamHI* site in the plasmid was engineered so that after the ligation, the myc epitope sequence in the insert was fused in-frame with the *RRD1* gene. The function of the epitope-tagged gene was tested by its ability to replace a plasmid-borne wild-type *RRD1* in an *rrd1 rrd2* strain (Y869). It was able to restore the growth of the *rrd1 rrd2* strain to a level that was indistinguishable from that of an *rrd2* allele, suggesting that the tagged gene was fully functional. The *RRD2(myc)₁₃* gene was created in a similar way. Briefly, the region of *RRD2* corresponding to positions -680 to the stop codon was PCR amplified from yeast genomic DNA and cloned into pRS314 between the *KpnI* and *BamHI* sites. A sequence for 13 tandem repeats of the myc epitope was then placed at the end of the gene as described for the *RRD1* gene. The *RRD2(myc)₁₃* gene was found to be fully functional based on its ability to support the growth the *rrd1rrd2* cells.

Gene Deletion. The *RRD1* gene was disrupted by replacing the region between the two *HindIII* sites in the gene with *HIS3*. The *RRD2* gene was disrupted by replacing the *BglIII*-*NheI* region of the gene with *HIS3*. The plasmid-borne deletions were introduced into Y663 by one-step gene replacement (Rothstein, 1991). The resulting strains were sporulated and tetrads were dissected. His⁺ haploid progeny were isolated from the tetrads of the diploid strains and the *rrd1::HIS3* and *rrd2::HIS3* deletions were verified by PCR. Strain Y845 was created by crossing Y841 (*rrd1::HIS3*) and Y844 (*rrd2::HIS3*). On introducing pRS416-*RRD1* plasmid, the cells of strain Y845 were sporulated and dissected. His⁺ Ura⁺ progeny were isolated from tetrads that showed 2:2 segregation for His⁺. The resulting strain, which bears the *rrd1 rrd2* double deletion and plasmid pRS416-*RRD1*, was found to be sensitive to 5-fluoroorotic acid, indicating that the double deletion was lethal in this strain background and required pRS416-*RRD1* for survival. Strains Y850, Y851, and Y869 were derived from Y845 by replacing pRS416-*RRD1* with pRS314-*RRD1(myc)₁₃*, pRS314-*RRD2(myc)₁₃*, and pRS415-*SSD1-v1*, respectively, by using plasmid shuffling (Sikorski and Boeke, 1991).

Epitope-tagging of Cdc55. A PCR fragment containing a truncated *cdc55* gene (+658 to stop codon) was cloned into pRS406 between the *XhoI* and *BamHI* sites. A triple hemagglutinin (HA) cassette was then inserted at the *BamHI* site that was excised from pFA6a-3HA-*TRP1* by digesting with *BamHI* and *BglIII* (Longtine *et al.*, 1998). The resulting plasmid contained a truncated 3' triple HA-tagged *cdc55* gene followed by an *ADHI* termination sequence. The plasmid was linearized by digestion with *PstI* at the site internal to the truncated *cdc55* gene and transformed into Y850 and Y852 for integration at the *CDC55* locus. The resulting strains, Y929 and Y930, contained a truncated *cdc55* gene along with a triple HA-tagged full-length *CDC55* gene.

Construction of Glutathione S-Transferase (GST)-Expression Plasmids. pRS416-*ADHp-GST* and pRS416-*ADHp-GST-SIT4* were constructed previously, which contained the *GST* and *GST*-fused *SIT4* genes under control of an *ADHI* promoter, respectively (Wang *et al.*, 2003). The plasmids were transformed into Y850 for coexpression with the myc-tagged *RRD1* gene.

Coimmunoprecipitation and GST Pull-Down

Preparation of Cell Extracts. Yeast cells (Y850) expressing either pRS415-*ADHp-GST* or pRS415-*ADHp-GST-SIT4* were grown in appropriate medium to early log phase. Cells were collected, washed once with ice-cold lysis buffer (50 mM Tris Cl, pH 7.4, 50 mM NaF, 5 mM EDTA, 1 mM dithiothreitol [DTT], and 5% glycerol), resuspended in the same buffer containing protease inhibitor cocktails (Roche Diagnostics), and lysed by vortexing with glass beads. Cell lysates were diluted onefold with wash buffer containing 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 300 mM NaCl, 1 mM DTT, 1% Triton X-100, and protease inhibitors. Insoluble cell debris was removed by centrifugation at 12,000 × g for 15 min. Protein concentration of the lysates was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

Immunoprecipitation. To precipitate myc epitope-tagged Rrd proteins, 15 μ l of anti-myc (9E10)-conjugated protein A beads (Santa Cruz Biotechnology) was added to 1 ml of cell extract (1 mg/ml). After incubation at 4°C for 3 h, the beads were washed three times with wash buffer, once with 20 mM Tris-Cl, pH 7.4, and boiled for 5 min in 70 μ l of 2× SDS sample buffer. Immunoprecipitation with anti-Tap42, -Sit4, and -HA antibodies has been described previously (Wang *et al.*, 2003).

GST Pull-Down with Yeast Extracts. An aliquot (1 ml) of cell extract containing 2 mg of protein was incubated with 60 μ l of glutathione beads (Amersham Biosciences, Piscataway, NJ) for 1 h at 4°C. After the incubation, the beads were washed twice with wash buffer and twice with phosphate-buffered saline (PBS). The bound proteins were then eluted from the beads by incubating for 10 min in 1 ml of PBS containing 10 mM glutathione (Sigma-Aldrich). The beads were removed by centrifugation, and the supernatant was then incubated with 20 μ l of anti-Tap42 antibody-conjugated protein A beads for 2 h at 4°C with gentle rocking. The beads were then washed twice with PBS, once with 20 mM Tris-Cl, pH 7.4, and boiled for 5 min in 70 μ l of 2× SDS sample buffer. Western blot analysis was then performed to detect the presence of GST-Sit4, Tap42, and Rrd1-myc in the precipitates by using anti-GST (1:2000), Tap42 (1:1500), and myc (9E10 at 1:1000) antibodies as described previously (Wang *et al.*, 2003).

GST Pull-Down with Bacterial Extracts. The *RRD1* gene was PCR amplified, cloned into pGEX-6P-1 vector (Amersham Biosciences), and expressed in BL21(DE3) cells. *TAP42* was expressed from pET22b vector as described previously (Jiang and Broach, 1999). Extracts from cells expressing *GST* or *GST-RRD1* fusion genes were incubated with glutathione beads for 5 min at room temperature. After washing three times with PBS, the coated beads were treated with 10 mg/ml bovine serum albumin in PBS buffer for 30 min and then incubated with bacterial extracts expressing Tap42 for 1 h at 4°C. The beads were washed four times with wash buffer containing 50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 2 mM DTT, and 1% Triton X-100 and once with 20 mM Tris-Cl, pH 7.4. The washed beads were boiled for 5 min in the presence of 2× SDS sample buffer. Western blotting was used to detect the GST, GST-Rrd1, and Tap42 proteins bound to the beads.

RESULTS

Physical interactions between the Rrd proteins and the catalytic subunits of PP2A and 2A-like phosphatases have been demonstrated previously (Mitchell and Sprague, 2001; Feller *et al.*, 2003; Van Hoof *et al.*, 2004). However, because these phosphatases exist in both Tap42-dependent and -independent forms (Di Como and Arndt, 1996), it is not clear which form of the phosphatases is the direct target of the Rrd proteins. As a first proof that the Rrd proteins were part of the Tap42-phosphatase complexes, we reexamined the interactions between the Rrd proteins and the phosphatases in our strain background. Accordingly, we immunoprecipitated myc epitope-tagged Rrd1 and Rrd2 from cell extracts and determined the presence of Sit4 and PP2Ac in the precipitates by Western blot analysis. As shown in Figure 1, we found that both phosphatases copurified with the Rrd proteins (Figure 1A, lane 2 and 3). Interestingly, the interaction between the Rrd proteins and the phosphatases seems to be highly selective, because we found that Sit4 coprecipitated almost exclusively with Rrd1 (Figure 1A, middle, compare lanes 2 and 3) and Pph21 mainly with Rrd2 (Figure 1A, bottom, compare lanes 2 and 3). These findings demonstrate a distinct feature for Rrd1 and Rrd2 in phosphatase interaction. Nevertheless, a small amount of Sit4 was found to be copurified with Rrd2 (Figure 1A, lane 3). A slight increase in the level also was observed when *RRD2* was overexpressed or *RRD1* was deleted from the cells (Zheng and Jiang, unpublished observation). These findings suggest a partially overlapped binding activity between Rrd1 and Rrd2. Similarly, a small portion of Pph21 was found to be associated with Rrd1 (Figure 1A, lane 3). However, in this case, some of Pph21 was believed to be from the Tpd3-Pph21-Rrd1 complex (see below). The drastic difference in the amount of the phosphatases copurified with Rrd1 and Rrd2 was not due to their expression levels, because we found that the levels of

Figure 1. Interaction of the Rrd proteins with Sit4 and Pph21. Yeast cells (Y162) expressing *HA-Pph21* were transformed with pRS314 (lane 1), pRS314-*RRD1(myc)*₁₃ (lane 2), or pRS314-*RRD2(myc)*₁₃ (lane 3). (A) Interaction of the myc-tagged Rrd proteins (Rrd-myc) with Sit4 and the triple HA epitope-tagged Pph21 (HA-Pph21) was examined by coimmunoprecipitation by using anti-myc antibody (9E10). Sit4 and HA-Pph21 copurified with either Rrd1-myc (lane 2) or Rrd2-myc (lane 3) were shown by Western blotting with anti-Sit4 and -HA (12CA5) antibodies. (B) Expression levels of the Rrd proteins Sit4 and HA-Pph21 in the cell extracts used in A were analyzed by Western blotting.

the phosphatases were the same in all the cells used in this experiment (Figure 1B).

On determination of the specific interactions between the Rrd proteins and the two different phosphatases, we assessed the association between the Rrd proteins and Tap42 by coimmunoprecipitation. As shown in Figure 2, when Tap42 antibody was used to precipitate Tap42 from cell extracts, both Rrd1 and Rrd2 were found in the precipitates (Figure 2A, lanes 2 and 3). Conversely, when myc antibody was used to purify the myc epitope-tagged Rrd1 and Rrd2, Tap42 was found to be copurified with both Rrd1 and Rrd2 (Figure 2B, lanes 2 and 3). These results demonstrate that Rrd1 and Rrd2 physically interact with Tap42.

To confirm that the Rrd proteins were part of the Tap42-phosphatase complexes, we examined the existence of the Tap42-Sit4-Rrd1 ternary complexes by using two sequential pull-down assays. In the first pull-down, GST-Sit4, along with proteins associated with it, was precipitated with glutathione beads. In the second pull-down, the proteins eluted from the glutathione beads were precipitated with anti-Tap42 antibody. As shown in Figure 3, in the first pull-down both Tap42 and Rrd1 were found to be copurified with GST-Sit4 (lane 4) but not with GST alone (lane 3). In the

Figure 2. Rrd1 and Rrd2 physically associate with Tap42. The interaction of Tap42 with either Rrd1-myc or Rrd2-myc was examined by coimmunoprecipitation. (A) Anti-Tap42 antibody was used to precipitate extracts from cells expressing a control vector (Y847, lane 1), pRS314-*RRD1(myc)*₁₃ (Y846, lane 2), and pRS314-*RRD2(myc)*₁₃ (Y848, lane 3). The presence of the myc-tagged Rrd proteins (Rrd-myc) in the precipitates was determined by Western blotting. (B) Anti-myc (9E10) antibody was used to precipitate myc-tagged Rrd1 and Rrd2 from the same cell extracts used in A. The presence of Tap42 in the myc precipitates was shown by Western blotting.

Figure 3. Rrd1 forms a ternary complex with Tap42 and Sit4. Extracts from cells expressing the *RRD1(myc)*₁₃ gene (Y850) together with either a GST (lane 1) or GST-SIT4 gene (lane 2) were precipitated with glutathione beads. The presence of Rrd1-myc and Tap42 in the GST (lane 3) or GST-Sit4 (lane 4) precipitates was detected by Western blotting. Proteins released from the beads were then immunoprecipitated with anti-Tap42 antibody, and the presence of GST-Sit4 and Rrd1-myc in the Tap42 precipitates was determined by Western blotting (lanes 5 and 6).

second pull-down, both GST-Sit4 and Rrd1 were found in the Tap42 precipitates (lane 6). Should the three proteins form independent dimers between them, only GST-Sit4 or Rrd1 but not both would occur in the Tap42 precipitate. The occurrence of both GST-Sit4 and Rrd1 in the precipitate indicated that the three proteins were in the same complex. As a control, GST was not found in the Tap42 precipitate (lane 5), indicating that Tap42 bound only to Sit4 but not GST.

Previously, it has been reported that Rrd1 interacts with Sit4 by binding to its C-terminal catalytic domain (Mitchell and Sprague, 2001). On the other hand, our recent work shows that Tap42 binds to the N-terminal region of Sit4 (Wang *et al.*, 2003). It is thus possible that phosphatases may act as a bridge to bring Tap42 and the Rrd proteins into the same complex. To test this notion, we examined the interaction of Tap42 with Rrd1 and Rrd2 in cells lacking of either Sit4 or Pph21 and Pph22. As shown in Figure 4, a significantly decreased amount of Tap42 was found to be copurified with Rrd1 in cells lacking Sit4 compared with wild-type cells (Figure 4A, compare lanes 1 and 2). Similar reduction in the amount of Tap42 coprecipitated with Rrd2 also was observed in the *pph21 pph22* cells in comparison with that in the wild-type cells (Figure 4A, compare lanes 3 and 4). The diminished interaction between Tap42 and the Rrd proteins in the absence of the phosphatases suggests that the phosphatases play a significant role in facilitating the interaction. Nevertheless, these results also reveal that Tap42 is able to interact with the Rrd proteins independently of the phosphatases. To rule out the possibility that the reduced interaction between Tap42 and Rrd1 in the absence of Sit4 was mediated by other 2A-like phosphatases, we examined the interaction by using an *in vitro* GST pull-down assay. As shown in Figure 4, we found that bacterially expressed recombinant Tap42 was copurified with a GST-Rrd1 fusion protein but not with GST (Figure 4C, compare lanes 4 and 5), suggesting that Tap42 possessed an intrinsic binding activity toward Rrd1.

Figure 4. Tap42 is able to interact with the Rrd proteins independent of the phosphatases. Plasmids pRS314-*RRD1*(myc)₁₃ or pRS314-*RRD2*(myc)₁₃ were introduced into the *sit4* (Y397), *pph21 pph22* (Y531) mutants as well as their isogenic wild-type alleles (Y062 and Y661). Extracts made from these strains were immunoprecipitated with anti-myc antibody. (A) Amounts of Tap42 copurified with Rrd1-myc from the wild-type (lane 1) and *sit4* (lane 2) cell extracts were examined by Western blotting. Similarly, the levels of Tap42 copurified with Rrd2 from the wild-type (lane 3) and *pph21 pph22* (lane 4) cell extracts were analyzed by Western blotting. (B) The levels of Tap42 in extracts of wild-type (lanes 1 and 3), *sit4* (lane 2), and *pph21 pph22* (lane 4) cells were examined by Western blotting. (C) Beads coated with GST (lane 1) and GST-Rrd1 (lane 2) were incubated with extracts from cells expressing Tap42 (lane 3). Tap42 bound to GST (lanes 4) and GST-Rrd1 (lane 5) was detected by Western blotting.

The mammalian PTPA was shown to act on the AC dimeric core of the PP2A holoenzyme (Cayla *et al.*, 1990, 1994). We thus determined whether the AC dimer of PP2A in yeast was also a target of the Rrd proteins. Accordingly, we immunopurified myc epitope-tagged Rrd1 and Rrd2 from cell extracts and examined the precipitates for the presence of Tpd3, the only A subunit of PP2A in yeast. As shown in Figure 5, Tpd3 was found to be coprecipitated with both Rrd1 and Rrd2, suggesting that the A subunit was able to interact with both proteins. However, Cdc55, one of the B subunits of PP2A, was not found in the precipitates. This result indicates that the Rrd proteins interact with the AC dimeric core but not the holoenzyme. In addition, the finding that both Rrd1 and Rrd2 interact with Tpd3 indicates that at least some of the Pph21 copurified with Rrd1 shown in Figure 2 was from the Tpd3-containing complex.

Interestingly, the amount of Tpd3 copurified with Rrd1 was found to be similar to that copurified with Rrd2, indicating that Rrd1 and Rrd2 were equally effective in their interaction with the AC dimer. This is in contrast to their interaction with Pph21 shown in Figure 1, in which case Pph21 was mainly associated with Rrd2, and only a small fraction was in complex with Rrd1. This discrepancy suggests that the most of Pph21 copurified with Rrd2 shown in Figure 1 was in the Tap42-containing complex. To confirm this notion, we compared the amount of Rrd2 associated with Tap42 with that associated with Tpd3. This was done by immunoprecipitating Tap42 and Tpd3 by using excessive

Figure 5. The Rrd proteins interact with the A subunit of PP2A. Extracts from cells expressing either *RRD1*(myc)₁₃ (Y929) or *RRD2*(myc)₁₃ (Y930) were precipitated with anti-myc antibody. The presence of Tpd3 (middle) and Cdc55-HA (bottom) in the cell extracts (Ext) and the myc precipitates (IP) was analyzed by Western blotting.

amount of antibodies so that nearly all the antigens were precipitated from cell extracts (Figure 6A). As shown in Figure 6, the amount of Rrd2 copurified with Tpd3 was significantly less than that copurified with Tap42 (Figure 6, B and C). Similarly, significantly less Rrd1 was found to be associated with Tpd3 than with Tap42 (Figure 6D). These findings suggest that the Tap42-containing complexes are the major targets of the Rrd proteins.

Figure 6. The Tap42-phosphatase complexes are the major targets of the Rrd proteins. Extracts from cells expressing *RRD2*(myc)₁₃ (Y852) were precipitated with either anti-Tap42 or anti-Tpd3 antibody. (A) Levels of Tap42 (top) and Tpd3 (bottom) in the extracts (ext), supernatants (sup), and precipitates (IP) were analyzed by Western blotting. (B) Amounts of Rrd2-myc copurified with Tap42 and Tpd3 were compared by Western blot analysis. (C) Quantitative presentation of the Western blotting result shown in B. (D) Extracts from cells expressing *RRD1*(myc)₁₃ (Y850) were precipitated with either anti-Tap42 or anti-Tpd3 antibody. The amount of Rrd1-myc copurified with either Tap42 (lane 1) or Tpd3 (lane 2) was analyzed by Western blotting.

Figure 7. Interaction between Tap42 and the Rrd proteins is rapamycin sensitive. Yeast cells (Y162) expressing either *RRD1*(myc)₁₃ or *RRD2*(myc)₁₃ were treated with rapamycin (200 nM) or drug vehicle for 1 h. Tap42, Sit4 and HA-Pph21 were precipitated from extracts made from the treated or untreated cells with anti-Tap42 (A and B), -Sit4 (C), and -HA (D) antibodies. (A) Western blot analysis of Rrd1-myc (middle) and Sit4 (bottom) copurified with Tap42 (top). (B) Western blot analysis of Rrd2-myc (middle) and HA-Pph21 (bottom) copurified with Tap42 (top). (C) Western blot analysis of Rrd1-myc (bottom) copurified with Sit4 (top). (D) Western blot analysis of Rrd2-myc (bottom) copurified with HA-Pph21 (top).

The interaction of Tap42 with Sit4 and PP2Ac has been shown to be rapamycin sensitive (Di Como and Arndt, 1996). It is believed that rapamycin induces the release of the phosphatases from Tap42, and consequently their activation (Jacinto *et al.*, 2001). Therefore, upon demonstration that the Rrd proteins were part of the Tap42-phosphatase complexes, we sought to determine whether the interaction of Tap42 with the Rrd proteins also was rapamycin sensitive. As shown in Figure 7, rapamycin treatment significantly reduced the amount of Rrd1 (Figure 7A, middle) and Rrd2 (Figure 7B, middle) copurified with Tap42. Interestingly, the extent of reduction was similar to the decrease in the amount of Tap42 associated with the phosphatases (Figure 7, A and B, bottom), indicating that rapamycin induced the release of both the Rrd proteins and the phosphatases from Tap42. Conversely, rapamycin did not seem to affect the interaction between the Rrd proteins and the phosphatases, because the amounts of Sit4 and Pph21 copurified with Rrd1 and Rrd2, respectively, from the treated and untreated cells were similar (Figure 7, C and D, compare lanes 1 and 2). These findings indicate that although the interaction between Tap42 and the Rrd proteins is rapamycin sensitive, the interaction between the Rrd proteins and phosphatases is not. It is thus evident that rapamycin treatment causes the release of the Rrd-phosphatase dimer from Tap42.

Release of Sit4 from Tap42 accompanies activation of the phosphatase, which is essential for rapamycin-induced dephosphorylation of many factors downstream of the Tor proteins, including Gln3, a GATA transcription factor that is involved in nitrogen catabolism in yeast (Beck and Hall, 1999; Cardenas *et al.*, 1999; Bertram *et al.*, 2000). On finding that the Rrd proteins were released together with the phosphatases, we asked whether these proteins were required for the rapamycin-induced activ-

Figure 8. Rrd proteins are required for rapamycin-induced activation of the Sit4 phosphatase. Wild-type (Y661), *rrd1* (842), *rrd2* (Y843), *rrd1 rrd2* (Y874), and *sit4* (Y397) cells expressing a myc-tagged *Gln3* gene were treated with or without rapamycin (200 nM) for 30 min. Cell extracts were subjected to SDS-PAGE followed by Western blotting with anti-myc antibody. The dephosphorylation of Gln3 is shown by the faster migrating bands on the SDS-PAGE.

ity of the phosphatases. Accordingly, we examined the dephosphorylation of Gln3 in response to rapamycin treatment in cells lacking either Rrd1 or Rrd2 or both. As published previously (Cardenas *et al.*, 1999; Bertram *et al.*, 2000) and as shown in Figure 8, rapamycin induced dephosphorylation of Gln3, which was characterized by the appearance of a faster migrating band on SDS-PAGE (compare lanes 1 and 2). The dephosphorylation of Gln3 was absent in cells lacking Sit4 (compare lanes 9 and 10). Rapamycin caused a full dephosphorylation of Gln3 in cells without Rrd2 (compare lanes 2, 5, and 6) and a partial dephosphorylation in cells lacking Rrd1 (compare lanes 2, 3, and 4). However, rapamycin failed to induce Gln3 dephosphorylation in cells lacking both Rrd1 and Rrd2 (compare lanes 7 and 8). These results indicate that the Rrd proteins are required for rapamycin-induced activation of Sit4. The absence of Sit4 activation in the *rrd1 rrd2* double mutant and the partial activation of it in the *rrd1* single mutant seem to indicate a partially overlapped function between Rrd2 and Rrd1 in supporting Sit4 activation.

As mentioned in the introduction, the *RRD1* and *RRD2* genes are characterized by the rapamycin resistance associated with cells lacking either gene (Rempola *et al.*, 2000). Because the *rrd1* and *rrd1 rrd2* mutants displayed defect in Sit4 activation in the presence of rapamycin (Figure 8), we asked whether Sit4 activity was required for the drug resistance trait associated with the *rrd1* and *rrd2* cells. We thus examined the rapamycin sensitivity of the *sit4 rrd1* and *sit4 rrd2* double mutants. As published previously (Rempola *et al.*, 2000) and as shown in Figure 9A, the *rrd1*, *rrd2* and *rrd1 rrd2* double deletions were found to be resistant to rapamycin. The drug resistance of the mutants seemed to correlate reversely with the Sit4 activity, with the *rrd1 rrd2* double mutant showing the least activity (Figure 8) and the most resistance (Figure 9A, compare the growth of the treated and untreated cells). Surprisingly, the *sit4 rrd1* and *sit4 rrd2* double mutants were found to be as sensitive to rapamycin as the *sit4* single mutant. This finding indicates that the *sit4* deletion is epistatic to both the *rrd1* and *rrd2* mutants. On the other hand, we found that the *sit4* deletion barely affected the resistance of the *gln3* deletion to rapamycin. The drug treatment reduced the growth of both the *gln3* and *gln3 sit4* cells by 1 order (Figure 9B, compare the growth of the cells in the presence and absence of the drug). The epistatic interaction of *gln3* with *sit4* is in accordance with the fact that Gln3 acts downstream of Sit4. Together, our findings suggest that the rapamycin resistant trait of the *rrd* mutants requires Sit4.

Figure 9. Rapamycin resistance of the *rrd1* and *rrd2* mutants is dependent upon Sit4. (A) Mid-log phase cells of wild-type (Y062), *rrd1* (Y884), *rrd2* (Y885), *rrd1 rrd2* (Y871), *sit4* (Y397), *rrd1 sit4* (Y886), and *rrd2 sit4* (Y887) strains were subjected to a series of 10-fold dilutions. The cells were spotted on YPD plates containing either 100 nM of rapamycin (YPD + Rap) or drug vehicle alone (YPD). Plates were photographed after incubated at 30°C for 48 h. (B) Rapamycin resistance of the wild-type (Y062), *gln3* (Y275), *sit4* (Y397), and *gln3 sit4* (Y964) strains was assayed as described in A.

DISCUSSION

PTPA was originally identified by its *in vitro* activity to stimulate the phosphotyrosyl phosphatase activity of the dimeric core of PP2A (Cayla *et al.*, 1990, 1994). Previous studies in yeast have revealed strong genetic interactions between the genes for the yeast PTPA and those encoding PP2Ac and the Sit4 phosphatase (Rempola *et al.*, 2000; Van Hoof *et al.*, 2000, 2001; Mitchell and Sprague, 2001). Those genetic results are further supported by the recent findings that the Rrd proteins and the catalytic subunits of PP2A and 2A-like phosphatases physically interact with each other, suggesting that the yeast PTPA may exert its activity *in vivo* by regulating phosphatases (Mitchell and Sprague, 2001; Fellner *et al.*, 2003; Van Hoof *et al.*, 2004). However, because PP2A and 2A-like phosphatases exist in multiple forms (Di Como and Arndt, 1996), the direct targets of PTPA in cells are largely unknown. In this study, we present evidence showing that the yeast PTPA, the Rrd proteins, is part of the Tap42-phosphatase complexes and that its activity is required for the function of the complexes (Figure 10). The Tap42-phosphatase complexes are a group of novel form of 2A and 2A-like phosphatases that play a major role in Tor-mediated signaling in yeast (Zabrocki *et al.*, 2002). The finding that the Rrd proteins are part of these complexes assigns a role for both Rrd proteins in the Tor signaling pathway and offers an explanation for the rapamycin resistance phenotype associated with the *rrd1* and *rrd2* cells. Interestingly, despite the sequence similarity between Rrd1 and Rrd2, these two proteins seem to have distinct binding specificity, because we find that Rrd1 specifically targets the Tap42-Sit4 complex and that Rrd2 interacts mainly with the Tap42-PP2Ac complex (Figure 10). Recently, Rrd1 has been found to associate also with Pph3 and Ppg1, the two 2A-like phosphatases in yeast (Van Hoof *et al.*, 2004), which supports a

Figure 10. Rrd proteins are part of the Tap42-phosphatase complexes. The Rrd proteins exist mainly in the Tap42-containing complexes, including the Tap42-Sit4-Rrd1 and Tap42-PP2Ac-Rrd2 complexes. Rapamycin causes release of the phosphatase-Rrd dimer from Tap42.

notion that Rrd1 specifically targets 2A-like phosphatases. These discrete roles for Rrd1 and Rrd2 are consistent with the distinct phenotypes associated with the *rrd1* and *rrd2* mutants (Rempola *et al.*, 2000; Van Hoof *et al.*, 2000, 2001; Mitchell and Sprague, 2001). However, although Sit4 is mainly associated with Rrd1, a small portion of the protein is associated with Rrd2 (Figure 1). This observation suggests that Rrd2 possesses a weak binding activity for the Tap42-Sit4 complex. Similarly, a fraction of Pph21 is associated with Rrd1, although some of Pph21 is believed to come from the complex formed between Rrd1 and the AC dimeric core. Together, these findings suggest a partially overlapped function between Rrd1 and Rrd2, which may explain the synthetic lethality of the *rrd1 rrd2* double mutant. Consistent with this view, we find that inactivation of Rrd1 only partially impairs the Sit4 activity for Gln3 dephosphorylation in the presence of rapamycin, whereas inactivation of both Rrd1 and Rrd2 nullified it (Figure 8).

In addition to the Tap42-phosphatase complexes, the Rrd proteins interact with the dimeric core of PP2A (Figure 5). However, the AC dimer does not seem to be the major target of the Rrd proteins. This is evident based on the amount of the Rrd proteins associated with Tpd3, which is significantly less than that associated with Tap42. The significance of the Rrd-AC complex is unclear. It may represent an intermediate that is required for the assembly of the holoenzyme. However, the finding that the PP2A holoenzyme assembles normally in the absence of the Rrd proteins argues against this possibility (Fellner *et al.*, 2003). Alternatively, the Rrd-AC complex may represent a novel form of PP2A.

Although our study demonstrates that the Tap42- and Tpd3-containing phosphatase dimers are the targets of the Rrd proteins, it is not clear whether these complexes are the only ones. A comparison of the amount of the Rrd proteins copurified with Tap42 with that left in the supernatant in a coimmunoprecipitation assay revealed that ~10–20% of the Rrd proteins was associated with Tap42, and conceivably, even less with Tpd3 (Zheng and Jiang, unpublished observation). This finding suggests that the Rrd proteins may

have other functions independent of Tap42 and Tpd3. However, because the efficiency in copurification depends not only on the amount but also on the binding affinity of the two interacting proteins, the coprecipitation result may not reflect precisely the amount of the proteins involved in the complex.

Despite the known *in vitro* phosphotyrosyl phosphatase activator activity of the Rrd proteins, the finding that these proteins are part of the Tap42–phosphatase complexes does not support a role for them in stimulating phosphotyrosyl phosphatase activity *in vivo*, because there is no indication that any of the known targets of the Tap42-associating phosphatases undergo tyrosylphosphorylation. The absence of Sit4-dependent dephosphorylation of Gln3 in the *rrd1 rrd2* double mutant cells indicates that the Rrd proteins are required for Sit4 activity, suggesting the Rrd proteins as positive regulators for the phosphatase. Nevertheless, although inactivation of the Rrd proteins confers rapamycin resistance to yeast cells, inactivation of Sit4 renders the cells hypersensitive (Rohde *et al.*, 2004). These distinct responses to rapamycin indicate that inactivation of the Rrd proteins does not nullify Sit4 activity. In support of this, we find that in the absence of Sit4, neither *rrd1* nor *rrd2* mutant is resistant to rapamycin (Figure 9), suggesting that the drug resistance trait of the mutant cells depends on the catalytic activity of Sit4. It is thus likely that failure to dephosphorylate Gln3 in the *rrd1 rrd2* mutant cells in response to rapamycin treatment is caused by changes in substrate specificity but not by loss of the catalytic activity of Sit4. In this view, the Rrd proteins act as allosteric regulators that control the substrate specificity of the phosphatases to which they associate. A recent finding that the PP2Ac isolated from cells depleted of both Rrd1 and Rrd2 displays an altered substrate specificity as well as a reduced stability is in accordance with this notion (Fellner *et al.*, 2003). It is thus plausible that the role of the Rrd proteins in regulating the Tap42-associated phosphatases mimics that of the B type subunits in regulating the AC dimeric core of PP2A.

In yeast, the Tap42–phosphatase complexes are major targets of the Tor-mediated signaling and are involved in Tor-mediated gene expression (Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000; Duvel and Broach, 2004). Inactivation of the Tor proteins by rapamycin treatment results in the release of phosphatases from Tap42, which is concomitant with their activation (Di Como and Arndt, 1996; Beck and Hall, 1999). This correlation has led to the suggestion that Tor proteins elicit their function by repressing phosphatase activity via a Tap42-dependent mechanism. Despite of this, inactivation of either Sit4 or PP2Ac, two major Tap42-associating phosphatases, does not confer yeast cells rapamycin resistance (Jiang and Broach, 1999), and in the case of Sit4 inactivation, it renders the cells more sensitive to the drug (Rohde *et al.*, 2004). In contrast, inactivation of factors that regulate the Tap42–phosphatase complexes is often found to confer rapamycin resistance. For instance, inactivation of Tip41, a negative regulator of Tap42–Sit4 interaction, confers rapamycin resistance (Jacinto *et al.*, 2001). Similarly, rapamycin resistance is associated with cells deleted for Tpd3 and Cdc55, which regulate the phosphorylation levels of Tap42 (Jiang and Broach, 1999). These findings strongly suggest that the drug resistance phenotype arises from alterations in substrate specificity of the phosphatases rather than loss of the catalytic activity. Our finding that the rapamycin-resistant trait of the *rrd* mutants depends on Sit4 activity is in accordance with this notion. The findings that the Rrd proteins physically interact with Sit4 and regulate its activity support the epistatic relationship of *sit4*

with *rrd1* and *rrd2* and argue against the possibility that the hypersensitivity to rapamycin of *sit4* nonspecifically supercedes the resistant trait of the *rrd* mutants. It is thus likely that in the absence of the Rrd proteins, or any other regulatory proteins, alterations in substrate specificity of Sit4 and PP2Ac cause aberrant dephosphorylation pattern of many downstream targets, allowing bypassing, at least partially, the requirement of the Tor proteins, and thereby conferring yeast cells rapamycin resistance. On the other hand, inactivation of Sit4 may leave many downstream factors of the phosphatase in their phosphorylated state, which may diminish the fitness of the cells and cause hypersensitivity to rapamycin. The finding that inactivation of Gln3 and Gcn2, two downstream targets of Sit4, is able to suppress the hypersensitivity of the *sit4* cells is in agreement with this notion (Figure 9A; Rohde *et al.*, 2004).

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