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Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast

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Tor proteins, homologous to DNA-dependent protein kinases, participate in a signal transduction pathway in yeast that regulates protein synthesis and cell wall expansion in response to nutrient availability. The anti-inflammatory drug rapamycin inhibits yeast cell growth by inhibiting Tor protein signaling. This leads to diminished association of a protein, Tap42, with two different protein phosphatase catalytic subunits; one encoded redundantly by *PPH21* and *PPH22*, and one encoded by *SIT4*. We show that inactivation of either Cdc55 or Tpd3, which regulate Pph21/22 activity, results in rapamycin resistance and that this resistance correlates with an increased association of Tap42 with Pph21/22. Furthermore, we show Tor-dependent phosphorylation of Tap42 both *in vivo* and *in vitro* and that this phosphorylation is rapamycin sensitive. Inactivation of Cdc55 or Tpd3 enhances *in vivo* phosphorylation of Tap42. We conclude that Tor phosphorylates Tap42 and that phosphorylated Tap42 effectively competes with Cdc55/Tpd3 for binding to the phosphatase 2A catalytic subunit. Furthermore, Cdc55 and Tpd3 promote dephosphorylation of Tap42. Thus, Tor stimulates growth-promoting association of Tap42 with Pph21/22 and Sit4, while Cdc55 and Tpd3 inhibit this association both by direct competition and by dephosphorylation of Tap42. These results establish Tap42 as a target of Tor and add further refinement to the Tor signaling pathway.

Keywords: protein phosphatase 2A/signaling/Tap42/Tor/yeast

Introduction

In the yeast *Saccharomyces cerevisiae*, two closely related proteins, Tor1 and Tor2, homologs of DNA-dependent protein kinases and phosphatidylinositol-3 lipid kinases, promote growth in response to nutrient availability (Kunz *et al*., 1993; Hall, 1996; Thomas and Hall, 1997). Tor2 activates Rom2, the exchange factor required for stimulating GTP binding to Rho1/Rho2 and subsequent organization of the actin cytoskeleton and activation of yeast Pkc1 (Schmidt *et al*., 1996, 1997; Helliwell *et al*., 1998). In addition, both Tor1 and Tor2 stimulate protein synthesis: inactivation of both of these proteins results in diminished initiation of protein synthesis (Barbet *et al*., 1996). In mammalian cells, Tor proteins phosphorylate eIF-4E-binding protein (4E-BP) and this phosphorylation alleviates inhibition of protein synthesis by 4E-BP (Beretta *et al*., 1996; Brunn *et al*., 1997b). While Tor proteins are also required for protein synthesis in yeast cells (Barbet *et al*., 1996), the mechanism of that stimulation is not known. Tor proteins also affect the stability of amino acid permeases in yeast, increasing the stability of some permeases, such as Tat2, and decreasing the stability of others, such as Gap1 and Put4 (Schmidt *et al*., 1998). This regulation is mediated by the Npr1 protein kinase, whose activity appears to be modulated by Tor (Vandenbol *et al*., 1990; Schmidt *et al*., 1998). Tor proteins possess kinase activity and, although evidence for lipid substrates has been presented (Helliwell *et al*., 1994; Cardenas and Heitman, 1995), the essential activity of Tor proteins is probably as protein kinases (Brunn *et al*., 1997a,b; Burnett *et al*., 1998).

In yeast, the anti-fungal and anti-inflammatory drug rapamycin binds to a prolyl isomerase, FKBP or Rbp1, and the complex of Rbp1 and rapamycin inhibits Tor-dependent activation of protein synthesis (Heitman *et al*., 1991b). Mutations of *TOR1* or *TOR2* that confer rapamycin resistance lie outside the domain for kinase function (Stan *et al*., 1994; Lorenz and Heitman, 1995). In addition, while rapamycin inhibits Tor-stimulated protein synthesis, it does not inhibit Tor-dependent actin organization and activation of Pkc1 (Zheng *et al*., 1995). These two observations suggest that rapamycin–Rbp complex does not inhibit kinase activity but rather blocks access of Tor to those substrates involved in promoting protein synthesis. One particular rapamycin-resistant mutation suggests that Tor proteins themselves may be regulated by phosphorylation. Mutation of Ser1972 in Tor1 (or Ser1975 in Tor2) to arginine or any other bulky amino acid yields resistance to rapamycin, while mutation of Ser1972 to alanine confers sensitivity to the drug (Stan *et al*., 1994; Lorenz and Heitman, 1995). One interpretation of this observation is that phosphorylation of Tor at this serine residue prevents binding of the rapamycin–Rbp complex to the protein. Consistent with this interpretation, Tor proteins are subject to phosphorylation *in vivo* (Brown *et al*., 1995; Scott *et al*., 1998).

As described below, protein phosphatases appear to play a role in Tor signaling activity. Two closely related genes, *PPH21* and *PPH22*, redundantly encode the major protein phosphatase 2A (PP2A) catalytic subunit in yeast (Sneddon *et al*., 1990). This catalytic subunit exists primarily in cells as a heterotrimeric complex with two other proteins, designated A and B, which regulate its activity. In yeast, *TPD3* encodes the only A subunit, and two distinct proteins, encoded by *CDC55* and *RTS1*, serve as alternative B subunits (Healy *et al*., 1991; van Zyl *et al*., 1992; Shu *et al*., 1997). Mutation of both *PPH21* and *PPH22* eliminates most of the PP2A activity in the cell and drastically reduces growth. Strains lacking *PPH21*,

PPH22 and a third related gene, *PPH3*, are completely inviable (Ronne *et al*., 1991). Mutation of *CDC55* yields defects in cytokinesis and morphology at lower temperatures, while mutation of *RTS1* results in growth defects at elevated temperatures (Healy *et al*., 1991; Shu *et al*., 1997). The phenotype of *tpd3* mutants resembles a composite of the phenotypes of *cdc55* and *rts1* strains, suggesting that Tpd3 is required for both Cdc55 and Rts1 function (van Zyl *et al*., 1992). Yeast cells contain a fourth 2A-like phosphatase catalytic subunit, encoded by *SIT4*, which performs functions in the cell distinct from those of Pph21/22 (Sutton *et al*., 1991). Sit4 normally associates with any one of three functionally redundant proteins Sap155, Sap185 or Sap190 (Luke *et al*., 1996). Sit4 complexed with any of the Sap proteins promotes progression through G_1, via regulation of G_1 cyclin production (Sutton *et al*., 1991; Fernandez-Sarabia *et al*., 1992). These results demonstrate that two distinct PP2A complexes, Pph21/22-based and Sit4-based, perform distinct essential functions in the cell.

Tap42, an essential protein, links Tor to the protein phosphatases (Di Como and Arndt, 1996). In growing cells, portions of Tap42 associate independently with both Sit4 and Pph21/22. This association of Tap42 with Pph21/22 does not require Tpd3 or Cdc55, and the association of Tap42 with Sit4 occurs independently of the Sap proteins. Tap42 does not associate with Sit4 and Pph21/22 in stationary phase cells or following treatment of cells with rapamycin (Di Como and Arndt, 1996). This latter observation, in conjunction with the fact that a mutant allele of *TAP42* confers rapamycin resistance, suggests that Tor proteins promote association of Tap42 with the two phosphatase catalytic subunits and that this association is a requisite step in the Tor-mediated signaling process.

In this study, we show that Tap42 is a substrate for Tor proteins both *in vivo* and *in vitro*. In addition, we show that mutation of *CDC55* or *TPD3* confers rapamycin resistance. Our analysis indicates that Cdc55 and Tpd3, while essential for normal growth in their own right, nonetheless antagonize the essential association of Tap42 with Pph21/22. Our results suggest that this antagonism involves both direct competition for association with the C subunit and dephosphorylation of the Tor-dependent phosphorylation of Tap42.

Results

Defects in PP2A regulation confer partial rapamycin resistance

The immunosuppressive macrolide rapamycin irreversibly arrests growth of wild-type yeast cells at the G_1 phase of the cell cycle. We observed that inactivation either of two regulatory subunits of PP2A, encoded by *TPD3* (A subunit) or *CDC55* (one of the B subunits), conferred rapamycin resistance to yeast cells (Figure 1A). Normally, cells lacking either Tpd3 or Cdc55 are thermosensitive and display a slower growth rate at 30°C than do wild-type cells (Healy *et al*., 1991; van Zyl *et al*., 1992). However, cells containing a deletion of either *TPD3* or *CDC55* were able to grow, albeit at a reduced rate, in the presence of 100 nM rapamycin, a concentration that completely inhibits growth of wild-type cells (Figure 1A). Similar phenotypes were observed with *tpd3-1* and *cdc55-1* mutant cells (data not shown). Both *tpd3*Δ strains and *cdc55*Δ strains were sensitive to 300 nM rapamycin, a concentration to which strains carrying the *TOR1*^{S1972R} allele are resistant. This result indicated that *tpd3*Δ or *cdc55*Δ mutant cells are partially resistant to the drug. In contrast, deletion of *RTS1*, a gene encoding another B subunit of PP2A, yielded cells that were as sensitive to rapamycin as wild-type cells (Figure 1A). Thus, activity conferred by Cdc55 and Tpd3 is required specifically for rapamycin sensitivity.

We also examined the rapamycin sensitivity of cells lacking catalytic subunits of PP2A. We found that deletion of either of the genes, *PPH21* or *PPH22*, encoding the major catalytic subunit (C subunit) of PP2A, did not confer rapamycin resistance (Figure 1B). Similarly, deletion of *SIT4* (in an *SSD1-v* background), which encodes a PP2A-related catalytic subunit, failed to produce a drug-resistant phenotype (Figure 1B). We also tested a haploid strain bearing deletions of both *PPH21* and *PPH22*. This strain lacked 80–90% of cellular PP2A activity but was as rapamycin-sensitive as wild-type cells (data not shown). Thus, we concluded that a defect in the regulation of PP2A, rather than a global decrease in PP2A activity, confers rapamycin resistance.

PP2A defects do not alter the binding of rapamycin–Rbp1 to Tor

Rapamycin acts by forming a complex with a cytoplasmic protein, Rbp1, and this complex in turn binds to Tor proteins to block their ability to stimulate protein synthesis (Heitman *et al*., 1991b). Disrupting the interaction between the rapamycin–Rbp1p complex and Tor proteins, either by deleting *RBP1* or by altering the rapamycin–Rbp1 binding domain of either of the Tor proteins, confers rapamycin resistance (Heitman *et al*., 1991a; Stan *et al*., 1994; Lorenz and Heitman, 1995). Hence, we tested the possibility that rapamycin resistance of *cdc55* or *tpd3* mutant strains resulted from interference in the interaction between the rapamycin–Rbp1 complex and the Tor proteins. This model was prompted by the observation that mutations changing Ser1972 in Tor1 or Ser1975 in Tor2 to any of a variety of bulky amino acids yielded both protein that failed to bind rapamycin–Rbp1 and strains that were resistant to rapamycin. In contrast, mutation of this serine residue to alanine failed to confer resistance (Stan *et al*., 1994; Lorenz and Heitman, 1995). Hence, a phosphate group introduced by phosphorylation of this serine residue would probably act like a bulky side chain and block the interaction required for rapamycin inhibition of Tor activity. Thus, in this model, dephosphorylation of Tor by PP2A would facilitate rapamycin–Rbp1 binding to Tor proteins, and loss of this specific PP2A activity would confer resistance.

To test this hypothesis, we constructed a strain that contained a mutant *TOR2*^{S1975A} as the sole *TOR* gene in the cell. Since the alanine residue cannot be phosphorylated, the mutant strain should remain sensitive to rapamycin regardless of the activity of PP2A, if Tor is the target of PP2A activity. However, inactivation of *CDC55* in this strain yielded rapamycin-resistant cells (Figure 2). A similar result was obtained by inactivation of *TPD3* in the same background (data not shown). These results suggest that PP2A does not affect the phosphorylation state of this serine residue *in vivo* and, consequently,

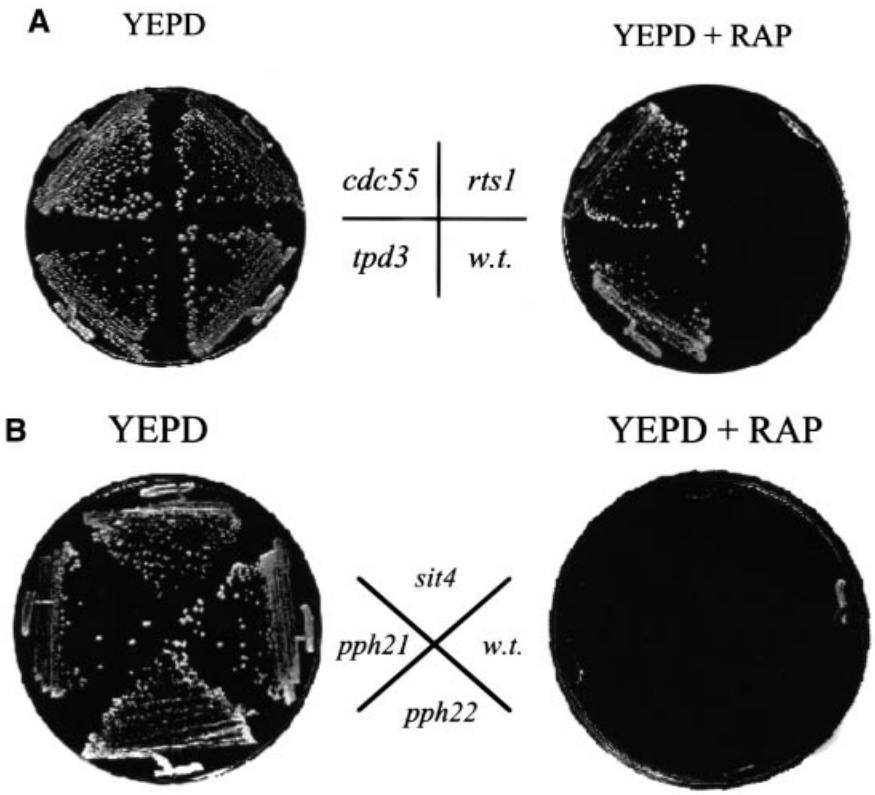

Fig. 1. Yeast mutants defective in protein phosphatase 2A regulation are rapamycin resistant. Strains W303-1A (*wt*), Y2487 (*tpd3*), Y2483 (*cdc55*) and Y2471 (*rts1*) (**A**) or strains W303-1A, Y2475 (*pph21*), Y2476 (*pph22*) and Y1361 (*sit4*) (**B**) were streaked on a YEPD plate (left) and a YEPD + rapamycin (100 nM) plate (right). Growth is shown after incubation at 30°C for 4 days.

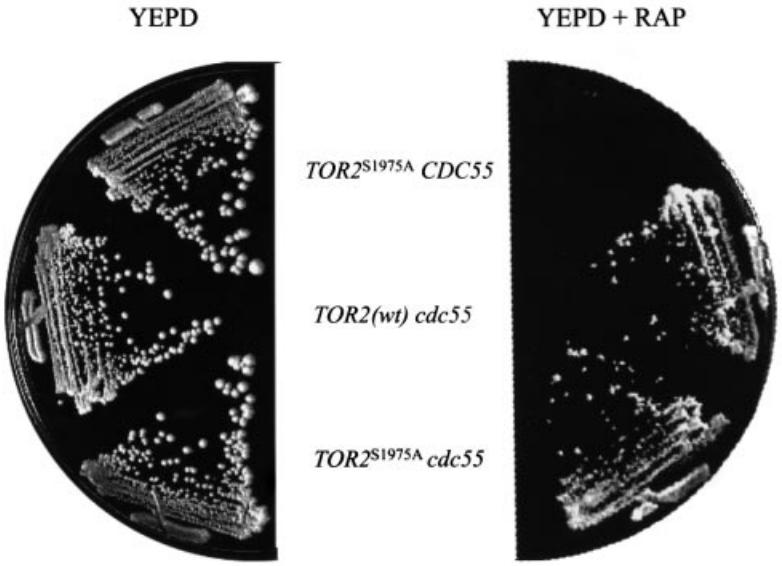

Fig. 2. Cdc55 acts downstream of Tor to confer rapamycin sensitivity. Strains Y2492 (*TOR2*^{1975A} *CDC55*), Y2483 (*TOR2 cdc55*) and Y2472 (*TOR2*^{1975A} *cdc55*) were streaked on a YPD plate (left) and a YPD + rapamycin (100 nM) plate (right). Growth is shown after incubation at 30°C for 4 days.

mutants of PP2A do not confer rapamycin resistance by altering the interaction of rapamycin–Rbp1 with Tor. Consistent with this interpretation, we found using a two-hybrid assay that rapamycin–Rbp1 complex could interact with the rapamycin–Rbp-binding domain of Tor as well in the absence of Cdc55 activity as its does in the presence of Cdc55 (data not shown). These results further suggest that mutation of Cdc55 or Tpd3 affects the Tor signaling pathway at a step downstream of the Tor proteins.

Cdc55 and Tpd3 compete with Tap42 for binding of the PP2A C subunits

An alternative explanation for the rapamycin resistance of *cdc55* and *tpd3* is that PP2A acts on a component of

the signaling pathway downstream of Tor. In this case, defects in PP2A could result in constitutive activation of that component, alleviating the need for Tor and thereby rendering yeast cells rapamycin resistant. One potential target of PP2A in the Tor signaling pathway is Tap42. Tap42 associates with the C subunits of yeast PP2A in a Tor signaling-dependent manner (Di Como and Arndt, 1996), suggesting that Tap42 acts downstream of Tor. In addition, deletion of *TPD3* enhances the association between Tap42 and Pph21, suggesting that Tpd3 might compete with Tap42 for binding of the C subunits. Accordingly, if association of Tap42 with the PP2A C subunit mediates Tor signaling, then competition of Tpd3 and Cdc55 with Tap42 for that association could account for the rapamycin resistance of *tpd3* and *cdc55* strains.

To test whether Tpd3 and Tap42 compete for binding to the PP2A C subunit, we first asked whether Tap42 co-exists with Tpd3 and Cdc55 in the same PP2A holoenzyme complex. Accordingly, we constructed strains containing genes encoding a c-Myc epitope-tagged Tap42 and a hemagglutinin (HA)-tagged Pph21 (Y2481) or Pph22 (Y2482) as the sole copies of the *TAP42* and *PPH21*/*PPH22* genes. Cell extracts prepared from the strains were incubated with anti-Tpd3, anti-c-Myc or anti-HA epitope antibodies. The precipitates were then analyzed by Western blot using antibodies against different components of PP2A complex. As published previously (Di Como and Arndt, 1996) and as shown in Figure 3, anti-HA antibodies immunoprecipitated not only HA-tagged Pph21 and Pph22, but also Tpd3, Cdc55 and Tap42 (Figure 3A). However, while anti-Tpd3 antibodies precipitated Tpd3 as well as Pph21, Pph22 and Cdc55, the anti-Tpd3 antibodies did not precipitate Tap42 (Figure 3B). Similarly, anti-Myc antibodies immunoprecipitated c-Myc-tagged Tap42, Pph21 and Pph22, but not Tpd3 or Cdc55 (Figure 3C). These results indicate that binding of Tap42 and Tpd3/Cdc55 to the C subunit are mutually exclusive, consistent with the observation that decreased levels of Tpd3 or Cdc55 increase the association of Tap42 with the PP2A C subunit (Di Como and Arndt, 1996).

To evaluate the functional significance of enhanced association of Tap42 with the PP2A C subunit, we examined the phenotypes of different strains overexpressing Tap42. As shown in Figure 4, we found that overexpression of Tap42 in wild-type cells promoted only a slightly enhanced resistance to rapamycin. However, Tap42 overexpression in either a *tpd3* or a *cdc55* strain provided an ~10-fold increase in rapamycin resistance over that observed in the absence of overexpression. Thus, conditions promoting increased association of Tap42 with PP2A C subunit also stimulate increased rapamycin resistance.

Overexpression of Tap42p enhances other phenotypes of *cdc55* strains in addition to rapamycin resistance. As reported previously (Healy *et al*., 1991) and as shown in Figure 5, *cdc55* deletion cells show defects in cytokinesis, often yielding multinucleate elongated cells with irregular constrictions along the length of the cell. Overexpression of Tap42 increases the penetrance (a greater proportion of elongated cells) and severity (more extensive elongation and multiple elongated buds from a single cell) of this phenotype. Thus, Tap42 appears to compete with Cdc55 and Tpd3 for binding to Pph21/22 and to exacerbate the phenotypes of *cdc55*.

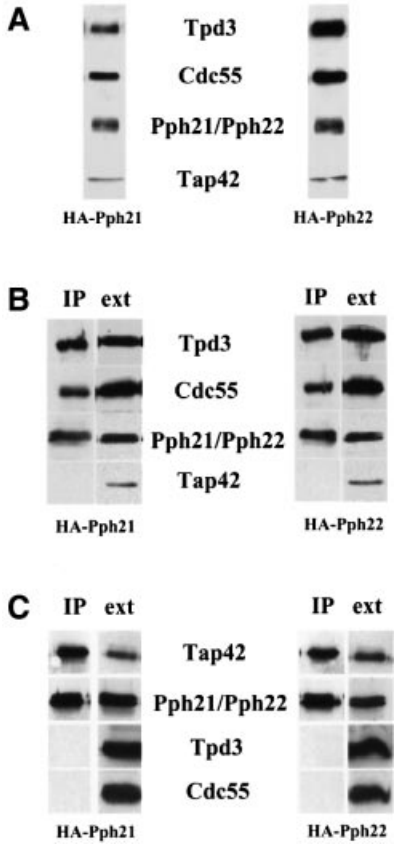

Fig. 3. Mutually exclusive binding of Tap42 and Tpd3/Cdc55 to the Pph21/22 catalytic subunit. Clarified extracts of strains Y2481 {W303-1A *tap42::HIS3 pph21::PPH21*$(HA)_3$ [pRS415-*TAP42*$(HA)_2$]} (left lanes) and Y2482 {W303-1A *tap42::HIS3 pph22::PPH22*$(HA)_3$ [pRS415-*TAP42*$(HA)_2$]} (right lanes) were immunoprecipitated with anti-HA epitope monoclonal antibodies (**A**), anti-Tpd3 polyclonal antibodies (**B**) or anti-c-Myc monoclonal antibodies (**C**). Samples of the immunoprecipitates ('IP' lanes) and of the extracts prior to precipitation ('ext' lanes) were fractionated by SDS–PAGE, transferred to nitrocellulose membranes and probed separately with anti-Tpd3, anti-Cdc55, anti-c-Myc (specific for Myc-tagged Tap42) and anti-HA (specific for HA-tagged Pph21 or Pph22) antibodies. Only that portion of each blot corresponding to the indicated protein is shown.

Tor kinase phosphorylates Tap42p

While competition between Tap42 and the PP2A regulatory subunits may explain in part the rapamycin-resistant phenotype of *cdc55* and *tpd3* strains, it does not explain the role of Tor signaling in controlling the interaction between Tap42 and the C subunits. Previous observations have suggested that this interaction requires Tor signaling: conditions that activate the Tor pathway promoted association of Tap42 with the C subunits. In contrast, depletion of nutrients or inactivation of Tor by addition of rapamycin resulted in disassociation of Tap42 from the C subunits (Di Como and Arndt, 1996).

To understand how Tor signaling controls the interaction of Tap42 with the C subunits, we investigated whether Tap42 is phosphorylated in a Tor-dependent fashion. Accordingly, we constructed a strain that contained a gene encoding a c-Myc-tagged Tap42 as the only copy of the *TAP42* gene. Since this strain was viable, the tagged construct fulfilled Tap42's essential activity in the cell. This strain was labeled with ^{32}P and the tagged Tap42 was precipitated from the cell extract using anti-c-Myc

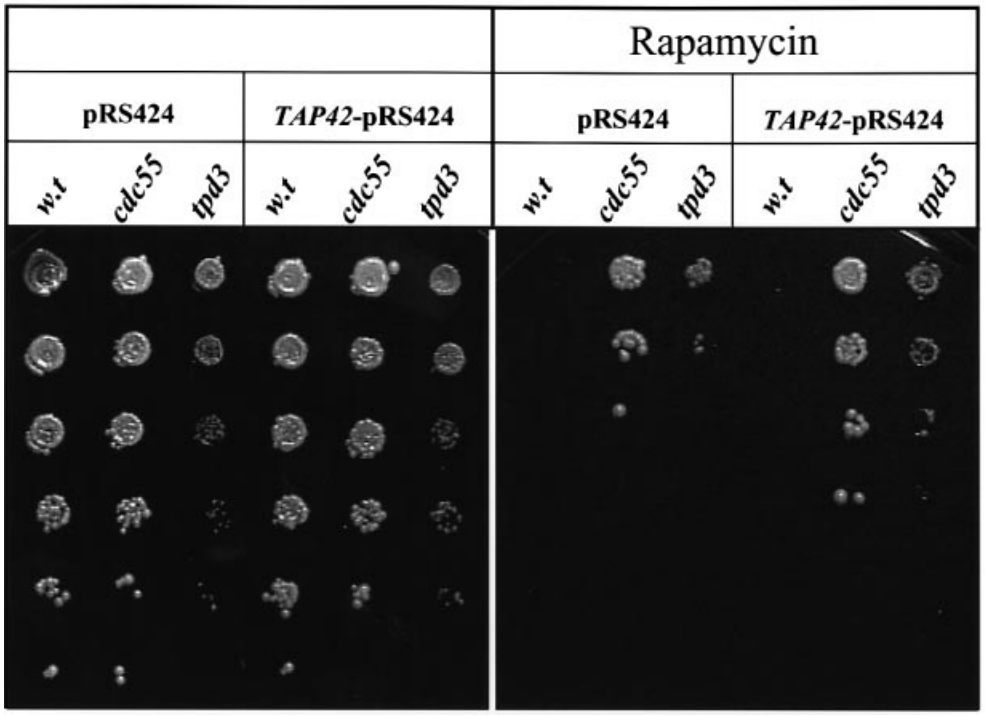

Fig. 4. Overexpression of Tap42 enhances rapamycin resistance of *tpd3* and *cdc55* mutant strains. Ten-fold serial dilution of logarithmically grown cells were spotted onto –Trp plates containing no rapamycin (left) or 100 nM rapamycin (right). Growth is shown after incubation at 30°C for 3 days in the absence of rapamycin or for 4 days in the presence of rapamycin.

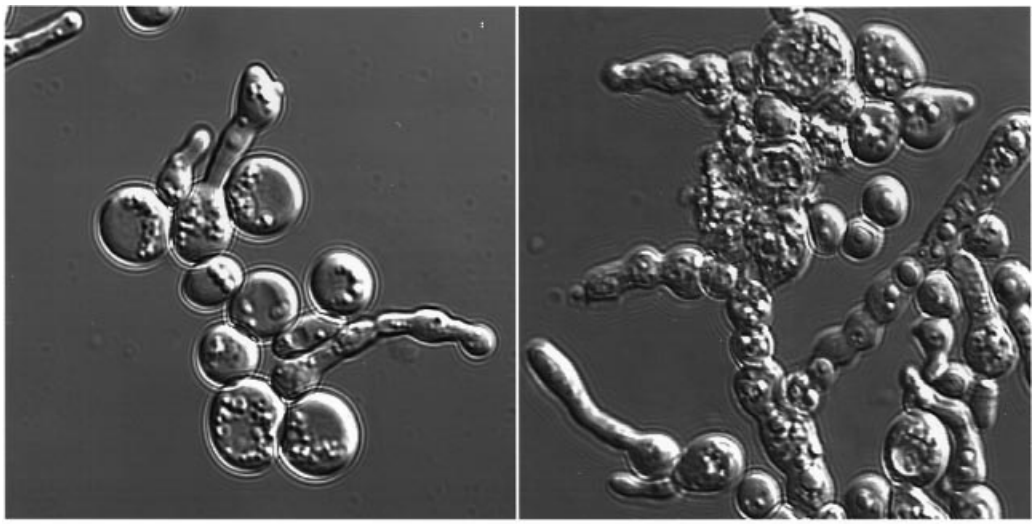

Fig. 5. Overexpression of Tap42 exacerbates the morphological defects of a *cdc55* mutant. DIC photomicrographs of *cdc55* (Y2483) and *cdc55* [pRS424-*TAP42*] (Y2491) cells grown in YEPD at 23°C.

antibodies. As is shown in Figure 6, ^{32}P label was incorporated into Tap42 in cells growing in the absence of rapamycin but not in cells treated with rapamycin (Figure 6A, lanes 2 and 3). This indicated that the incorporation of label into Tap42 is Tor dependent. Since Western blot analysis demonstrated that the amount of Tap42 in the two different cell extracts is essentially equivalent (Figure 6B, lanes 2 and 3), we concluded that the difference in the ^{32}P incorporation results from a difference in the level of phosphorylation of the protein under the two conditions. To confirm that the inhibition of phosphorylation of Tap42 by treatment of cells with rapamycin was a consequence of the inactivation of Tor, we introduced a rapamycin-resistant *TOR* mutation into the strain and labeled the cells in the presence of rapamycin. Under these conditions, we found that the incorporation of ^{32}P into Tap42 was equivalent to that seen in the absence of rapamycin (Figure 6A, lane 4). These results demonstrated that Tap42 is a phosphorylated protein and that phosphorylation is dependent upon Tor.

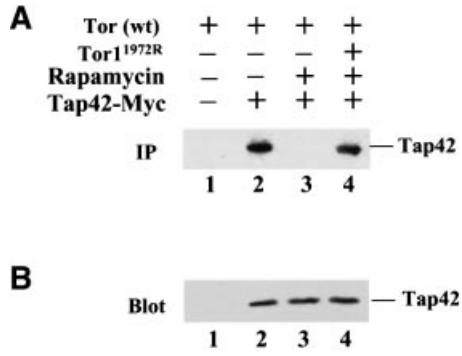

Fig. 6. *In vivo* Tor-dependent phosphorylation of Tap42. Strains W303-1A (*TAP42*; lane 1), Y2474 [*TAP42*(c-Myc)$_2$; lanes 2 and 3] and Y2491 [*TAP42*(c-Myc)$_2$ *TOR1*^{S1975R}; lane 4] were labeled with ^{32}P for 3 h in the presence (lanes 3 and 4) or the absence (lanes 1 and 2) of 500 nM rapamycin. Clarified extracts of the labeled cells were either immunoprecipitated with anti-c-Myc 9E10 ascites and fractionated by SDS–PAGE (**A**) or fractionated directly on SDS–PAGE, transferred to nitrocellulose and probed with anti c-Myc 9E10 ascites (**B**). Shown are the autoradiographs of the fractionated immunoprecipiates and the ECL-developed Western blot.

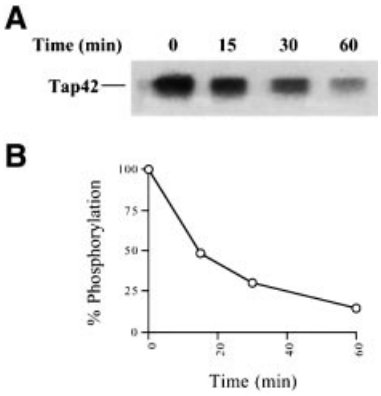

Fig. 7. Rapamycin treatment causes a rapid dephosphorylation of the phosphorylated Tap42 *in vivo*. Cells of strain Y2474 were labeled with ^{32}P in the absence of rapamycin for 3 h and chased in YEPD medium containing 500 nM rapamycin. Cells were removed at the indicated time points and lysed. Clarified extracts of the labeled cells were immunoprecipitated with anti-c-Myc 9E10 ascites, fractionated on SDS–PAGE and transferred to nitrocellulose membrane. The incorporation of ^{32}P was visualized by autoradiography (**A**) and quantitated using a phosphoimager (**B**). Shown is the average of two independent experiments.

To confirm that the effect of rapamycin on Tap42 phosphorylation was a direct consequence of inactivation of Tor kinase activity, we examined the time course of Tap42 dephosphorylation following rapamycin treatment. We first labeled yeast cells with ^{32}P for 3 h and then transferred labeled cells into fresh medium containing 500 nM rapamycin. Aliquots of cells were removed at different time points and lysed. c-Myc-tagged Tap42 was immunopurified from the cell extracts using anti c-Myc antibodies and the incorporation of ^{32}P into Tap42 was visualized by autoradiography. As shown in Figure 7, the amount of phosphorylated Tap42 decreased by ~50% by 15 min after addition of rapamycin, and most (80–90%) of the phosphorylated Tap42 disappeared within 60 min. The decrease in phosphorylation was not due to the decrease in the amount of Tap42, since the level of Tap42 protein remained essentially unchanged during the 1 h incubation in the presence of rapamycin (data not shown).

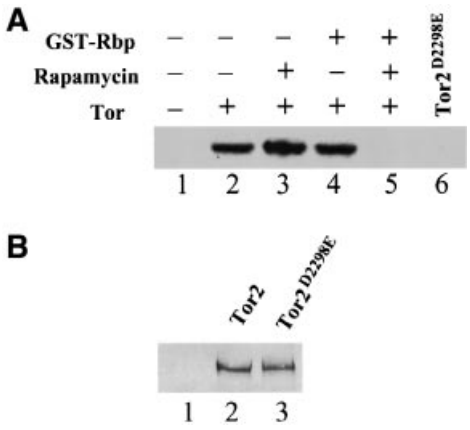

Fig. 8. Immunopurified Tor2 phosphorylates recombinant Tap42 *in vitro*. (**A**) Recombinant Tap42 was incubated for 1 h at 30°C with [γ-^{32}P]ATP and kinase buffer with addition of an immunoprecipitate obtained with anti-HA antibodies of Y2493 cells (*TOR2*; lane 1), Y2470 cells [*TOR2*(HA)$_3$; lanes 2–5] or Y2497 cells [*TOR2*^{D2298E}(HA)$_3$; lane 6]. Reactions in lanes 3 and 5 also contained 10 μM rapamycin, and the reaction in lanes 4 and 5 contained 10 μg of GST–Rbp1. After incubation, the extracts were fractionated by SDS–PAGE and the fractionated extracts transferred to nitrocellulose. Shown is an autoradiogram of the nitrocellulose filter. (**B**) Western blot of the immunoprecipitated Tor2 proteins. Anti-HA precipitates from Y2493 cells (TOR2; lane 1), Y2470 cells [*TOR2*(HA)$_3$; lane 2] and Y2497 cells [*TOR2*^{D2298E}(HA)$_3$; lane 3] were separated by 6% SDS–PAGE, transferred to nitrocellulose membrane and blotted with anti-HA antibodies.

Thus, inactivation of Tor kinase by rapamycin treatment causes a rapid dephosphorylation of phosphorylated Tap42. Since Tor kinase is required for the association of Tap42 with the C subunits (Di Como and Arndt, 1996), our observation is consistent with the hypothesis that the phosphorylated form of Tap42, but not the dephosphorylated form, associates with the C subunits and, thus, Tor-dependent phosphorylation of Tap42 drives its association with the C subunit.

To confirm that Tor is the Tap42 kinase, we immunopurified Tor and tested its activity in an *in vitro* kinase assay. We first constructed a strain containing a gene encoding a triple HA epitope-tagged Tor2 as the only *TOR* gene. The HA-tagged Tor2 was then purified by immunoprecipitation using anti-HA antibodies and precipitates were assayed for kinase activity using recombinant Tap42 as substrate. We found that precipitates from cell extracts expressing HA-tagged Tor2 were capable of phosphorylating Tap42 (Figure 8A, lanes 2–4). Immunoprecipitates obtained with anti-HA antibodies from extracts of cells not expressing the HA-tagged Tor2 protein did not exhibit Tap42 kinase activity (Figure 8A, lane 1). Furthermore, pre-incubation of the HA-Tor2 immunoprecipitates with rapamycin plus GST–Rbp fusion protein completely eliminated the kinase activity toward Tap42 (Figure 8A, lane 5). Treating the immunoprecipitate with either rapamycin alone (lane 3) or GST–Rbp1 alone (lane 4) did not affect its kinase activity, consistent with previous observations that only as a complex with Rbp1 protein does rapamycin inhibit Tor (Heitman *et al*., 1991b; Lorenz and Heitman, 1995). To fortify our conclusion that Tap42 phosphorylation activity in the immunoprecipitates results from Tor kinase activity, we introduced a kinase-inactivating amino acid substitution (D2298E) into wild-type Tor2.

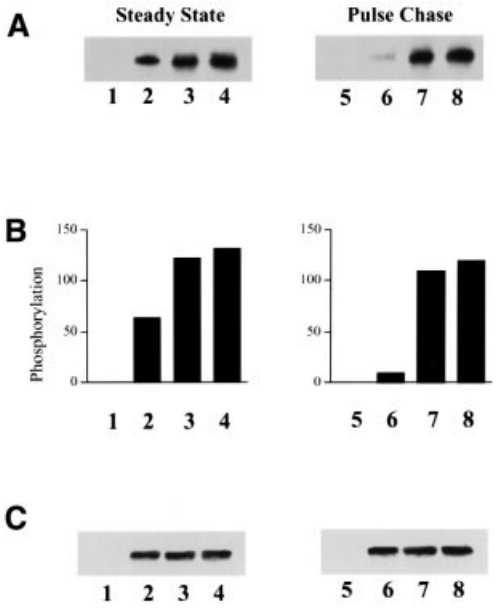

Fig. 9. Defects in PP2A enhance *in vivo* phosphorylation of Tap42. Yeast cells were labeled with ^{32}P for 3 h (lanes 1–4) and chased with unlabeled organic phosphate in the presence of 500 nM rapamycin for an additional 3 h (lanes 5–8). Tap42-Myc was immunoprecipitated with anti-c-Myc monoclonal antibodies from extracts of wild-type cells (W303-1A; lanes 1 and 5), wild-type cells expressing epitope-tagged Tap42 (Y2484; lanes 2 and 6), *cdc55* cells expressing epitope-tagged Tap42 (Y2485; lanes 3 and 7) or *tpd3* cells expressing epitope-tagged Tap42 (Y2486; lanes 4 and 8). Incorporation of ^{32}P into Tap42 was visualized by autoradiography (**A**) and was quantitated by phosphoimager analysis (**B**) (the average of two independent experiments is shown in arbitrary units). The amount of Tap42 in each of the cell extracts was determined by Western blot (**C**).

We found that, like the equivalent mutation in *TOR1*, which completely abolishes its function (Zheng *et al*., 1995), this mutation rendered Tor2 incapable of supporting cell growth (data not shown). Immunopurified $Tor2^{D2298E}$ mutant protein failed to phosphorylate recombinant Tap42 under the conditions in which the immunopurified wild-type Tor2 phosphorylated efficiently (Figure 8A, lanes 2 and 6). Western blot analysis revealed that the amount of precipitated mutant Tor2 protein was essentially equivalent to the amount of precipitated wild-type Tor2 (Figure 8B, lanes 2 and 3). These results indicate that a functional kinase domain in Tor2 protein is required for the Tap42 phosphorylation.

PP2A is required for the dephosphorylation of phosphorylated Tap42p

Having shown that Tap42 phosphorylation is dependent on Tor kinase, we asked whether PP2A is required for dephosphorylation of Tap42. To do so, we immunoprecipitated Tap42 from ^{32}P-labeled cells and compared the phosphorylation of Tap42 in strains defective in PP2A with that in wild-type cells. As shown in Figure 9, we found that the level of phosphorylated Tap42 after a 3 h labeling was significantly higher in *cdc55* (Figure 9A and B, lane 3) and *tpd3* mutant cells (Figure 9A and B, lane 4) than that in wild-type cells (Figure 9A and B, lane 2), even though the amounts of Tap42 in these strains were essentially equivalent (Figure 9C, lanes 2–4). This result suggests that defects in PP2A enhance the phosphorylation of Tap42, presumably due to reduced dephosphorylation activity that normally counterbalances the Tor-dependent phosphorylation activity. To test directly whether PP2A is required for dephosphorylation of phosphorylated Tap42, the labeled cells were shifted to chase medium and allowed to grow for an additional 3 h in the presence of rapamycin to inhibit further phosphorylation. Consistent with the results in Figure 7, we found that the level of phospho-Tap42 in wild-type cells declined significantly (Figure 9A and B, lane 6), even though the amount of protein remained the same (Figure 9C, compare lanes 2 and 6). In contrast, the phosphorylation level of Tap42 in the *cdc55* mutant (Figure 9A and B, compare lanes 3 and 7) and *tpd3* (Figure 9A and B, compare lanes 4 and 8) mutant cells remained high. This result demonstrates that phosphorylated Tap42 cannot be dephosphorylated efficiently in the absence of either *CDC55* or *TPD3*, suggesting that PP2A activity is required for the dephosphorylation of Tap42.

Discussion

Tap42 participates in the regulation of protein synthesis and amino acid permease turnover in response to Tor kinase activity (Di Como and Arndt, 1996; Schmidt *et al*., 1998). Mutational inactivation of Tap42 or inhibition of Tor proteins by rapamycin inhibits initiation of protein synthesis and stimulates turnover of Tat2 permease. In addition, certain alleles of Tap42 are rapamycin resistant, an observation that implicates Tap42 in the Tor signaling pathway and indicates that Tap42 acts downstream of Tor. Finally, both Tor and nutrient availability regulate the association of Tap42 with the phosphatase catalytic subunits Pph21/22 and Sit4, since either starvation or treatment of cells with rapamycin eliminates Tap42's association with these subunits. While no one has shown directly that Tap42 complexed with either Pph21/22 or Sit4 promotes initiation of protein synthesis or affects the activity of Npr1 kinase, the strict correlation between the biological effects of Tor and production of the Tap42–Pph21/22 and Tap42–Sit4 complexes provides circumstantial evidence that these complexes mediate Tor's effects.

The results presented here provide insight into the mechanism and regulation of Tap42 complex formation. We have shown here that Tap42 exhibits Tor-dependent, i.e. rapamycin-sensitive, phosphorylation *in vivo*. In addition, immunopurified wild-type Tor2, but not a kinase-dead mutant allele, possesses Tap42 kinase activity *in vitro*. While a distinct kinase that co-precipitates with Tor and that depends on Tor for its activity could be the actual Tap42 kinase, the most economical interpretation of our results is that Tor phosphorylates Tap42 directly. The coincidence of Tor-dependent phosphorylation of Tap42 and Tor-stimulated association of Tap42 with the phosphatase catalytic subunits suggests, although it does not prove, that these two events are linked causally, i.e. Tap42 phosphorylation could depend on its being bound to the phosphatase catalytic subunit. On the other hand, Tap42 association with the catalytic subunits could depend on phosphorylation catalyzed by Tor. Since we find that Tor can phosphorylate Tap42 *in vitro* in the absence of either phosphatase subunit, our results are more consistent with the latter possibility. Thus, a model in which nutrient availability stimulates the Tor kinases, which in turn phosphorylate Tap42 to promote its association with both

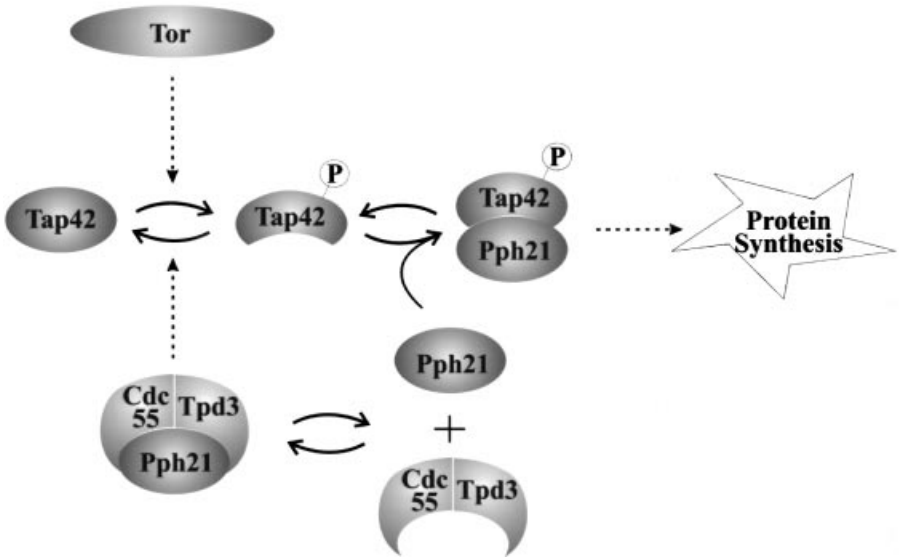

Fig. 10. Model for the role of PP2A in Tor regulation of protein synthesis. See text for details.

Sit4 and Pph21/22 to stimulate initiation of protein synthesis, can accommodate all available data.

We have shown that inactivation of Tpd3 or Cdc55 confers rapamycin resistance. Epistasis analysis demonstrated that these proteins act in the rapamycin-sensitive portion of the Tor signaling pathway downstream of the Tor proteins themselves. Consistent with this observation, we find that these proteins promote dephosphorylation of Tap42 *in vivo* and also that they compete with Tap42 for binding to Pph21/22. These observations prompted the model shown in Figure 10. In this model, Tor proteins phosphorylate Tap42, which facilitates its binding to free Pph21 or Pph22 catalytic subunit. This phosphorylation is reversed by the phosphatase activity of PP2A, comprised of Tpd3, Cdc55, and Pph21 or Pph22. In addition, formation of PP2A by association of Cdc55 and Tpd3 with Pph21/22 restricts the amount of free Pph21/22 subunit available to bind to Tap42. Thus, loss of Tpd3 or Cdc55 would increase the level of Tap42–Pph21/22 complex in the cell by increasing both the level of phosphorylated Tap42 and the availability of free Pph21/22. This would reduce the amount of Tor activity necessary to stimulate Tap42–Pph21/22 complex formation and thus render cells relatively rapamycin resistant.

Our model posits a positive role for the Tap42–Pph21/22 complex in the growth-promoting activity of the Tor pathway. Our results are not consistent with a model in which the primary role of Tap42 is as an inhibitor of Pph21/22 activity, by serving as a sink to diminish the level of PP2A, for instance. If this were the case, then *pph21 pph22* mutants should be rapamycin resistant, which we found not to be the case. In addition, since the amount of Tap42 in cells is significantly lower than the amount of Pph21/22, Tap42 could not eliminate Pph21/22 activity by stoichiometric binding to the C subunit. Thus, the function of Tap42 might be to redirect a portion of the Pph21/22 phosphatase activity to a substrate or substrates distinct from those recognized by PP2A. In this scenario, dephosphorylation of that substrate would be critical for stimulating growth.

A difficulty with the above model is that no obvious candidates for the substrates for a Tap42–Pph21/22 phosphatase present themselves. In mammalian cells, Tor protein regulates protein synthesis by promoting phosphorylation of 4E-BP to alleviate inhibition of eIF-4E by unphosphorylated 4E-BP (Beretta *et al*., 1996). In addition, Tor stimulates p70s6 kinase activity. In neither of these cases can dephosphorylation be invoked as a mediator of the growth-promoting signal initiated by Tor (Proud, 1996; Jefferies *et al*., 1997). In yeast, a homolog of 4E-BP has not been identified. However, eIF-4G, an essential initiation factor required for mRNA translation via the 5′ cap structure and whose association with eIF-4E is blocked by 4E-BP in mammalian cells, shows increased degradation following treatment of yeast cells with rapamycin (Berset *et al*., 1998). The degradation pathway is unknown, but if stabilization of eIF-4G is a result of Tor kinase activity, this could be a point of intervention of the Tap42–Pph21/22 phosphatase.

Tor regulation of permease activity also poses a quandary regarding the likely substrate of Tap42 activity. Tor appears to regulate amino acid permease activity by promoting phosphorylation, and consequently inhibition, of Npr1 kinase (Vandenbol *et al*., 1990; Schmidt *et al*., 1998). Inactivation of the Npr1 kinase stabilizes Tat2 permease and destabilizes Gap1 and Put4 permeases. Epistasis analysis places Tap42 upstream of Npr1 and downstream of Tor in this pathway (Schmidt *et al*., 1998), i.e. Tor activation of Tap42 results in increased phosphorylation of Npr1. Without invoking additional steps between Tap42 and Npr1, this observation is inconsistent with a role for Tap42 as a phosphatase.

An alternative model in which Tap42 bound to a PP2A C subunit or Sit4 converted it from a phosphatase to an anti-phosphatase could account for Tap42's role in Tor signaling. In this model, we hypothesize that the Tap42–Pph21/22 complex would protect substrate phosphoproteins from dephosphorylation by PP2A or other phosphatases. This would be consistent with the observation that activation of Tap42 (i.e. increased association of the protein with Sit4 and Pph21/22) correlates with increased phosphorylation of targets, Npr1 and 4E-BP, that lie

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain[a]	Genotype[b]	Source
Y1361[c]	α *sit4::HIS3 SSD1-v ura3-52 his3-1 leu2-3 trp1-1*	laboratory stock
Y1364[c]	α *cdc55-1 ura3-52 leu2-3,113*	laboratory stock
Y1459[c]	α *tpd3-1 hml::SUP4-o hmr::SUC2 sir4-1 leu2-3,112 his3D1 ade2-1 ura3-52*	laboratory stock
Y2470	**a** *tor1::HIS3 tor2::LEU2* [pRS314(*CEN4 TRP1*)-*TOR2*$(HA)_3$]	this study
Y2471	**a** *rts1::LEU2*	this study
Y2472	**a** *cdc55::LEU2 tor1::HIS3 tor2::URA3* [pRS314(*CEN4 TRP1*)-*TOR2*^{S1975A}]	this study
Y2473	**a** *cdc55::LEU2 tor1::HIS3*	this study
Y2474	**a** *tap42::HIS3 URA3::TAP42*$(c\text{-}Myc)_2$	this study
Y2475	α *pph21::HIS3*	this study
Y2476	**a** *pph22::TRP1*	this study
Y2477	**a** *tap42::HIS3* [pRS416(*CEN4 URA3*)-*TAP42*]	this study
Y2478	**a** *tap42::HIS3* [pRS415(*CEN4 LEU2*)-*TAP42*$(HA)_2$]	this study
Y2479	α *pph21::PPH21*$(HA)_3$	this study
Y2480	α *pph22::PPH22*$(HA)_3$	this study
Y2481	**a** *tap42::HIS3 pph21::PPH21*$(HA)_3$ [pRS415(*CEN4 LEU2*)-*TAP42*$(HA)_2$]	this study
Y2482	**a** *tap42::HIS3 pph22::PPH22*$(HA)_3$ [pRS415(*CEN4 LEU2*)-*TAP42*$(HA)_2$]	this study
Y2483	α *cdc55::LEU2*	this study
Y2484	α *tap42::HIS3 URA3::TAP42*$(c\text{-}Myc)_2$	this study
Y2485	**a** *tap42::HIS3 URA3::TAP42*$(c\text{-}Myc)_2$ *cdc55::LEU2*	this study
Y2486	α *tap42::HIS3 URA3::TAP42*$(c\text{-}Myc)_2$ *tpd3::LEU2*	this study
Y2487	**a** *tpd3::LEU2*	this study
Y2488[c]	***a*** *ura3-52 his3-1 leu2-3,112 trp1-1 lys2-801* [pRS424(2μ, *TRP1*)-*TAP42*]	this study
Y2489[c]	α *cdc55::LEU2 ura3-52 his3-1 leu2-3,112 trp1-1 lys2-801*[pRS424(2μ, *TRP1*)-*TAP42*]	this study
Y2490[c]	***a*** *tpd3::LEU2 ura3-52 his3-1 leu2-3,112 trp1-1 lys2-801*[pRS424(2μ, *TRP1*)-*TAP42*]	this study
Y2491	α *tap42::HIS3 URA3::TAP42*$(c\text{-}Myc)_2$ [pRS314(*CEN TRP1*)-*TOR1*^{S1972R}]	this study
Y2492	α *tor1::HIS3 tor2::URA3* [pRS314(*CEN TRP1*)-*TOR2*^{S1975A}]	this study
Y2493	***a*** *tor1::HIS3 tor2::LEU2* [pRS314(*CEN TRP1*)-*TOR2*]	this study
Y2494[c]	***a*** *cdc55::LEU2 ura3-52 his3-1 leu2-3,112 trp1-1 lys2-801*	this study
Y2495[c]	***a*** *tpd3::LEU2 ura3-52 his3-1 leu2-3,112 trp1-1 lys2-801*	this study
Y2496[c]	α *ura3-52 his3-1 leu2-3,112 trp1-1 lys2-801*	this study
Y2500	**a** *TOR1 TOR2* [pRS314(*CEN TRP1*)-*TOR2*^{D2998E}]	this study
KTD30	**a** *tor2::LEU2 tor1::HIS3* [YCp50(*CEN4 URA2*)-*TOR2*^{S1975R}]	K.Freeman

[a]Strains listed were derived from W303-1A (**a** *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*) or W303-1B (α *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*) unless otherwise indicated.
[b]Plasmids are indicated in square brackets. Yeast markers carried on each plasmid are listed in parentheses following the plasmid designation.
[c]Strains were derived from an S288C background.

downstream of Tap42 in the Tor signaling pathway (Beretta *et al*., 1996; Di Como and Arndt, 1996; Schmidt *et al*., 1998). In addition, this model would account for the fact that overexpression of Tap42 exaggerates the phenotypes of *cdc55* and *tpd3* mutants. This model is also consistent with the observation that α4, the mammalian homolog of Tap42, reduced the relative *in vitro* activity of the rabbit AC PP2A dimer toward phosphorylated phosphorylase a, a known substrate of PP2A (Murata *et al*., 1997). A precedent for such an anti-phosphatase activity comes from studies on 14-3-3 proteins, which bind to specific phosphoserine domains on proteins (Muslin *et al*., 1996) and which, in the case of Raf-1, can increases the lifespan of the phosphorylated serine *in vivo* (Thorson *et al*., 1998). It is noteworthy in this context that overexpression of 14-3-3 proteins in yeast confers an increased resistance to rapamycin (Bertram *et al*., 1998). While this hypothesis requires additional data for conformation, it does provide an economical explanation for the role of Tap42 in Tor signaling.

The Tor signaling pathway shows remarkable phylogenetic conservation. Mammalian cells possess a Tor kinase, structurally homologous to the yeast Tor kinases, that mediates T cell activation in response to cytokine stimulation (Thomas and Hall, 1997). As in yeast, this kinase is sensitive to rapamycin bound to FKBP, the homolog of Rbp1. In addition, Tor regulates initiation of protein synthesis (Beretta *et al*., 1996; von Manteuffel *et al*., 1996; Hara *et al*., 1998) and mammalian cells contain a Tap42 homolog, designated α4, which also exhibits rapamycin-sensitive binding to the PP2A C subunit (Murata *et al*., 1997; Chen *et al*., 1998). Thus, the observations on the role of Tor in yeast cells should provide direct insights into the corresponding process in mammalian cells.

Materials and methods

Strains, media and reagents

All strains used in this study are listed in Table I and are derived from W303-1A or W303-1B. Strain YKT301 is a gift from Dr Katie Freeman at SmithKline Beecham Pharmaceuticals. Yeast cells normally were grown at 30°C in YEPD or synthetic complete (SC) medium containing 2% glucose as a carbon source. For the ^{32}P labeling experiment, strains were grown in low-phosphate medium (LPM) containing 2% glucose (Rubin, 1973). Anti-c-Myc (9E10) ascites and anti-HA (12CA5) cell culture supernatants were obtained from the Princeton University Monoclonal Antibody Facility. Anti-Tpd3 and anti-Cdc55 polyclonal antibodies were raised in rabbits against recombinant GST–Tpd3 and GST–Cdc55, respectively.

Gene deletion

The *TPD3* gene was inactivated as described before (van Zyl *et al*., 1992). *CDC55* was inactivated by replacing the 1.5 kb *Bgl*II–*Nhe*I region of the gene with a PCR-generated 2.2 kb *Bgl*II–*Nhe*I fragment containing *LEU2*. *TAP42* was inactivated by replacing the 0.9 kb *Psh*AI–*Sna*BI region of the gene with a PCR-generated 1.1 kb *Psh*AI–*Sma*I fragment

containing *HIS3*. *PPH21* was disrupted by replacing the 0.8 kb *Eco*RI–*Mfe*I region of the gene with a PCR-generated 1.1 kb *Eco*RI–*Eco*RI fragment containing *HIS3*. *PPH22* was disrupted by replacing the 0.5 kb *Bsa*BI–*Eco*RV region of the gene with a PCR-generated 1.1 kb *Sma*I–*Sma*I fragment containing *TRP1*.

Epitope tagging

Epitope-tagged genes were constructed by *in vitro* site-directed mutagenesis using oligonucleotides containing coding sequences for either HA or Myc epitopes. Epitope-tagged Tap42 contains two tandem copies of c-Myc epitopes in place of the third amino acid of the protein. Epitope-tagged Tor2 contains a triple HA tag in place of the fourth and fifth amino acids of the protein. Epitope-tagged Pph21 and Pph22 contain a triple HA tag inserted in-frame between the first and the second amino acids of each protein. Triple HA epitope-tagged *PPH21* and *PPH22* were used to replace the wild-type chromosomal allele by two-step gene replacement (Guthrie and Fink, 1991).

Co-immunoprecipitation and immunoblot

Yeast cells were grown in YEPD medium to early log phase. Cells were then harvested, washed twice with 10 mM ice-cold sodium azide and converted to spheroplasts by a 45 min incubation in spheroplasting buffer (50 mM sodium phosphate, pH 7.5, 1.4 M sorbitol, 50 mM β-mercaptoethanol) containing 10 μg of Zymolyase T1000 per OD unit of cells. Spheroplasts were lysed with lysis buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 2% Triton X-100 and protease inhibitors). Cell lysates were diluted 4-fold with lysis buffer. Insoluble debris was removed by centrifuging for 15 min. A sample (3 mg) of the supernatant was incubated overnight on ice with either anti-Tpd3 antibody (10 μl) or anti-c-Myc ascites fluid (15 μl). The immune complexes were precipitated with protein A–beads (100 μl of a 50% protein A–Sepharose 4B slurry) during a 90 min incubation at 4°C. Beads were washed twice with lysis buffer containing 1% Triton X-100, twice with 100 mM Tris–HCl, pH 7.5, 300 mM NaCl and once with 10 mM Tris–HCl, pH 7.5. After the final wash, beads were boiled for 5 min in 70 μl of 2× SDS sample buffer. An aliquot (20 μl) of each sample was loaded onto a 10% polyacrylamide–SDS gel and subjected to electrophoresis. Samples were then transferred onto a nitrocellulose membrane and probed with antibodies against Tpd3 (1:2000 dilution), Cdc55 (1:2000 dilution), HA epitope (9E10, 1:1000) or c-Myc epitope (12CA5, 1:1000).

^{32}P-radiolabeling, immunoprecipitation and immunoblotting

Yeast cells were grown in LPM overnight and transferred to fresh LPM for an additional 3 h. Cells (10 OD_{600} U) were harvested and resuspended in 5 ml of LPM containing 3 mCi of $^{32}PO_4$ (Amersham). Cells were harvested after labeling for 3 h. For pulse–chase labeling experiments, labeled cells were washed twice with YEPD medium, resuspended in 10 ml of YEPD, split into two equal cultures to one of which was added 500 nM rapamycin and both culture grown for an additional 3 h. Cells from both cultures were harvested, washed extensively with ice-cold 10 mM sodium azide and converted into spheroplasts as described above. Spheroplasts were lysed by boiling for 5 min in the presence of 1% SDS. An aliquot (500 μg) of cell extracts was diluted 10-fold with phosphate-buffered saline (PBS) buffer containing 0.5% Triton X-100, 2 mg/ml bovine serum albumin (BSA) and protease inhibitors and centrifuged for 15 min to remove insoluble cell debris. The supernatant was incubated overnight on ice with 10 μl of 9E10 ascites fluid. The immune complexes were precipitated onto protein A–beads (100 μl of a 50% protein A–Sepharose 4B slurry) during a 90 min incubation at 4°C. Beads were washed once with PBS, twice with urea wash buffer (2 M urea, 100 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100) and twice with 1% β-mercaptoethanol. After the final wash, beads were resuspended in 70 μl of 2× SDS sample buffer and boiled for 5 min. Proteins were separated by electrophoresis on a 10% polyacrylamide–SDS gel and transferred to nitrocellulose membrane. Radiolabeled Tap42 was visualized by autoradiography and quantitated with a phosphoimager. Immunoblotting was performed using the same cell extracts. Proteins were separated on a 10% polyacrylamide–SDS gel, transferred to nitrocellulose membrane and probed with 9E10 ascites fluid (1:1000 dilution). Tap42 was detected by the ECL detection method (Amersham).

Preparation of recombinant Tap42 and GST–Rbp1

A PCR-generated *TAP42* open reading frame (ORF) was cloned into the *Nde*I and *Xho*I sites of pET22b expression vector (Novagen). The ORF sequence was fused in-frame at its 3′ end with a His_6 tag sequence on the vector. This vector was transformed into BL21(DE3) bacterial cells and expressed. Recombinant His-tagged Tap42 was purified using an Ni^{2+}-NTA–agarose column (Qiagen). The *RBP1* gene was cloned into pGEX vector and fused in-frame with the GST portion on the vector. This vector was introduced into BL21(DE3) cells and expressed. GST–Rbp1 fusion protein was purified using glutathione-coupled Sepharose beads.

Immunopurification of Tor2

Strain Y2470 was grown overnight to early log phase. Cell lysates were prepared as described in the co-immunoprecipitation experiment. An aliquot of 1 mg of lysate protein was then incubated at 4°C for 2 h with 3 μg of purified anti-HA epitope antibody (12CA5). Immune complexes were precipitated by incubating at 4°C for 90 min with protein A–Sepharose beads (50 μl of a 50% slurry). Precipitates were washed twice with lysis buffer containing 1% Triton X-100, twice with 100 mM Tris–HCl, pH 7.5, 300 mM NaCl, and twice with kinase buffer [10 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT)]. After the final wash, beads were resuspended in 25 μl of kinase buffer. The reaction was initiated by the addition of 25 μl of reaction mixture containing 1 mM ATP, 2.5 μCi of [γ-^{32}P]ATP and recombinant Tap42p (100 μg/ml). For the rapamycin-treated experiment, 10 μM rapamycin and 10 μg of purified GST–Rbp protein were added to protein A–beads and incubated at 30°C for 30 min prior to the addition of reaction mixture. After incubation for 1 h at 30°C, the reaction was terminated by adding 12 μl of 5× SDS sample buffer and boiling for 5 min. An aliquot of sample (20 μl) was fractionated by SDS–PAGE on a 10% polyacrylamide gel, and transferred to a nitrocellular membrane. ^{32}P incorporation was visualized by autoradiography.

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