Rapamycin activates Tap42-associated phosphatases by abrogating their association with Tor complex 1

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In *Saccharomyces cerevisiae*, the Tap42–phosphatase complexes are major targets of the Tor kinases in the rapamycin-sensitive signaling pathway. The immunosuppressive agent, rapamycin, induces a prompt activation of the Tap42-associated phosphatases, which is vitally important in Tor-mediated transcriptional regulation. However, the mechanism for the rapid phosphatase activation is poorly understood. In this study, we show that the Tap42–phosphatase complexes exist mainly on membrane structures through their association with Tor complex 1 (TORC1). Rapamycin abrogates this association and releases the Tap42–phosphatase complexes into the cytosol. Disassembly of the Tap42–phosphatase complexes occurs subsequently, following the release but at a much slower rate, presumably caused by Tap42 dephosphorylation. Release of the Tap42–phosphatase complexes from membrane structures also occurs when cells are deprived of nutrient. These findings suggest that the association of the Tap42–phosphatase complexes with TORC1 represents an important mechanism by which nutrient controls Tor signaling activity. In addition, our data support a model in which rapamycin acts not by inhibiting the kinase activity of Tor but by disrupting its interaction with downstream targets.

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Introduction

In the yeast *Saccharomyces cerevisiae*, the Tor1 and Tor2 kinases reside at the center of a signaling cascade that plays a vital role in regulation of cell growth in response to nutrient availability and conditions (Schmelzle and Hall, 2000). Tor-mediated signaling activity controls a wide range of growth-related cellular processes, including transcription, translation, autophagy, actin distribution and protein stability (Rohde *et al*, 2001; Powers *et al*, 2004). The immunosuppressive and anticancer agent rapamycin, in complex with

immunophilin FKBP12, specifically binds and inhibits the Tor kinases (Heitman *et al*, 1991). Inhibition of Tor by rapamycin induces physiologic changes resembling those under starvation condition, and consequently, ceases cell growth and arrests cell cycle progression at early G1 phase (Barbet *et al*, 1996). Both Tor1 and Tor2 participate in mediating G1 progression and the shared function is sensitive to rapamycin (Kunz *et al*, 1993). However, Tor2 possesses a unique function that is not sensitive to rapamycin (Zheng *et al*, 1995). This Tor2 unique function is involved in regulating polarized actin distribution during bud growth and plays an important role in coordinating cell morphogenesis with growth (Schmidt *et al*, 1996).

The Tor kinases elicit their shared function at least in part by regulating Tap42, a phosphatase 2A-associating protein (Di Como and Arndt, 1996). Tap42 is a conserved protein that has been shown to interact with the catalytic subunits of type 2A phosphatase (PP2Ac), including Pph21 and Pph22, and several 2A-like phosphatases, such as Sit4, Pph3 and Ppg1 (Di Como and Arndt, 1996; Wang *et al*, 2003). The interaction of Tap42 with the phosphatases is a dynamic process that is regulated by nutrient availability. In cells grown in nutrient-rich medium, Tap42 is found to associate with phosphatases, but in cells under poor nutrient conditions, Tap42 is free from phosphatases. However, even under optimal growth conditions, Tap42 associates only with a small portion of the phosphatases. For instance, only 5–10% of Sit4 and PP2Ac is found to associate with Tap42 in actively growing cells (Di Como and Arndt, 1996). How Tap42 selectively interacts with a subset of the phosphatases is not clear. Inhibition of Tor by nutrient deprivation or rapamycin treatment induces dissociation of Tap42 from the phosphatases, suggesting that Tor regulates the Tap42–phosphatase interaction in response to nutrient conditions (Di Como and Arndt, 1996; Wang *et al*, 2003). Tap42 is found to undergo Tor-dependent phosphorylation, and is phosphorylated when Tor is active, which correlates with its association with phosphatases. This correlation has led to the suggestion that Tor-dependent phosphorylation promotes the interaction of Tap42 with phosphatases (Jiang and Broach, 1999). Interestingly, dissociation of phosphatases from Tap42 is accompanied by dephosphorylation of many downstream targets of the Tor signaling pathway, such as Npr1, Ure2 and Gln3, indicating that release of the phosphatases from Tap42 causes their activation (Schmidt *et al*, 1998; Beck and Hall, 1999; Cardenas *et al*, 1999). Hence, it has been suggested that Tap42 acts as a phosphatase inhibitor, which binds and inhibits phosphatases in response to Tor signaling activity. Inactivation of Tor by rapamycin results in release of the phosphatases from Tap42 inhibition, and consequently, their activation (Jacinto *et al*, 2001).

Tor1 and Tor2 exist in two distinct multiprotein complexes termed Tor complex 1 (TORC1) and Tor complex 2 (TORC2) (Loewith *et al*, 2002; Wedaman *et al*, 2003). TORC1 mediates

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the rapamycin-sensitive branch of the Tor signaling pathway. It contains either Tor1 or Tor2, together with two highly conserved proteins, Kog1 and Lst8. TORC2 is responsible for the Tor2 unique function and is composed of Tor2 and several other components, including Lst8 and the Avo proteins, Avo1–3. All of the Tor-associating proteins, except Avo2, are essential for cell viability, suggesting that the integrity of the Tor complexes is required for the function of Tor1 and Tor2. Recently, additional components of these complexes have also been identified, including Tco89 and Bit64, two nonessential proteins, which are associated with TORC1 and TORC2, respectively (Reinke *et al*, 2004). Both TORC1 and TORC2 are found to associate with membrane structures located in regions proximal to the plasma membrane and within the interior of the cell (Kunz *et al*, 2000; Wedaman *et al*, 2003). However, the nature of these membrane structures remains unclear. Nor is it known how these complexes become membrane-bound, as none of the identified components possesses any features that may potentially mediate membrane association. It has been shown that rapamycin, in complex with FKBP, is able to bind to TORC1. Nevertheless, the integrity of the complex, as well as its membrane association, is undisturbed by the drug, suggesting that rapamycin interferes with Tor function by means other than disrupting TORC1 (Kunz *et al*, 2000; Loewith *et al*, 2002; Wedaman *et al*, 2003).

The finding that the Tor kinases exist in multiprotein complexes that reside on membrane structures suggests a complicated regulation mechanism for Tor activity, raising questions regarding how the Tor kinases signal their downstream targets in the context of the complexes and how rapamycin interferes with the signaling. In this study, we show that the Tap42–phosphatase complexes, the major effector of the Tor kinases in the rapamycin-sensitive signaling pathway, are physically associated with TORC1 on membrane structures in a nutrient-dependent and rapamycin-sensitive manner.

Results

A small portion of Tap42 is membrane-bound

To better understand how the membrane-bound TORC1 regulates Tap42 and its interaction with phosphatases, we analyzed the intracellular distribution of Tap42 by fractionation. Accordingly, cell extracts were partitioned into soluble and membrane fractions by centrifugation at 100 000 g. As shown in Figure 1A, the majority (~90%) of Tap42 was found in the soluble fraction (S₁₀₀), consistent with Tap42 being a soluble protein. However, a small portion of Tap42 (~10%) was found in association with the membrane fraction (P₁₀₀), which contains membrane structures and large protein particles. To determine the nature of the association, we treated the membrane fraction with various reagents that are known to disrupt either protein–protein or protein–lipid interaction. We found that treatment with 1 M NaCl partially disrupted the association of Tap42 with the membrane fraction, whereas treatment with 1% Triton X-100 or a combined treatment of both salt and detergent made Tap42 largely soluble (Figure 1B). As the partial sensitivity to both salt and detergent is often a characteristic of membrane-bound protein complex, the above result indicates that Tap42 in the

Figure 1 Tap42 associates with membrane structures. Extracts (Total) from exponentially growing cells (Y959) were partitioned into soluble (S₁₀₀) and membrane (P₁₀₀) fractions. (A) The presence of Tap42 in each fraction was analyzed by immunoblotting. (B) The membrane fraction was resuspended and treated with the indicated reagents for 30 min on ice followed by centrifugation at 100 000 g. The levels of Tap42 in the supernatant (S) and pellet (P) were analyzed by immunoblotting. “*” in this and other figures denotes a nonspecific protein.

membrane fraction may exist in a membrane-bound protein complex.

The membrane-bound Tap42 is associated with TORC1

Interestingly, the distribution of Tap42 in the supernatant and pellet after the membrane fraction was treated with salt or detergent or both is reminiscent of that of the membrane-bound Tor kinases treated with the same reagents (Wedaman *et al*, 2003). Given the fact that Tap42 is a target of the Tor kinases, it is thus possible that the membrane-associated Tap42 is in the same complex as the Tor kinases. To confirm this notion, we obtained soluble and membrane fractions from cell extracts, and analyzed the potential association between Tap42 and Tor2 in both fractions. As shown in Figure 2A, we found that most of Tor2 was associated with the membrane fraction (left panel), consistent with Tor2 being a peripheral membrane protein (Kunz *et al*, 2000). Upon solubilizing the membrane fraction with detergent, Tor2 was effectively co-immunoprecipitated with Tap42 (right panel). Like Tor2, Tor1 existed mainly in the membrane fraction (Figure 2B, left panel) and was co-precipitated with Tap42 (Figure 2B, right panel). These results suggest that the portion of Tap42 in the membrane fraction is associated with the Tor kinases.

As TORC1 contains either Tor1 or Tor2, the finding that Tap42 is associated with both proteins indicates that Tap42 is in complex with TORC1. To confirm this, we analyzed the association of Tap42 with Kog1, which is unique to TORC1, and with Avo1, which is TORC2 specific, by co-immunoprecipitation (Loewith *et al*, 2002; Wedaman *et al*, 2003). As shown in Figure 2C, Kog1 was found to be co-purified with Tap42 from the detergent-solubilized membrane fraction (lane 2). In contrast, Avo1 was absent from the Tap42 precipitate (Figure 2D, lane 2). These findings demonstrate that Tap42 is in complex with Kog1 but not with Avo1, confirming that Tap42 is a TORC1-associating protein.

The association of Tap42 with TORC1 is rapamycin sensitive

As Tap42 acts downstream of the Tor kinases in the rapamycin-sensitive pathway (Di Como and Arndt, 1996; Jiang

Figure 2 Tap42 is in complex with TORC1. (A) Extracts from cells expressing HA-Tor2 (BY2470) were fractionated into soluble (S₁₀₀) and membrane (P₁₀₀) fractions. The distribution of Tap42 and HA-Tor2 in the fractions was determined by immunoblotting (IB). Both fractions were treated with Triton X-100, and precipitated with (α-Tap42) or without (mock) anti-Tap42 antibody. The presence of Tap42 and HA-Tor2 in the precipitates was analyzed by immunoblotting (IP). (B) Extracts from cells expressing HA-Tor1 (Y990) were fractionated into soluble (S₁₀₀) and membrane (P₁₀₀) fractions, and the distribution of Tap42 and HA-Tor1 in the fractions was analyzed by immunoblotting (IB). Both fractions were treated with Triton X-100 and precipitated with (α-Tap42) or without (mock) anti-Tap42 antibody. The presence of Tap42 and HA-Tor1 in the precipitates was determined by immunoblotting (IP). (C) The membrane fraction (P₁₀₀) of the cells expressing HA-Kog1 (Y1032) was solubilized with Triton X-100, and precipitated with (α-Tap42) or without (mock) anti-Tap42 antibody. The presence of Tap42 and HA-Kog1 in the precipitate was analyzed by immunoblotting (IP). (D) The membrane fraction (P₁₀₀) of cells expressing HA-Avo1 (Y1031) was solubilized with Triton X-100 and precipitated with (α-Tap42) or without (mock) anti-Tap42 antibody. The presence of Tap42 and HA-Avo1 in the precipitate was analyzed by immunoblotting (IP). In panels C and D, the amount of P₁₀₀ in lane 1 represents 10% of the total input for the precipitation shown in lanes 2 and 3.

and Broach, 1999), we determined whether the association of Tap42 with TORC1 was susceptible to rapamycin inhibition. We first compared the amount of Tap42 in the membrane fractions before and after the cells were treated with rapamycin. As shown in Figure 3A, rapamycin treatment significantly reduced the amount of Tap42 in the membrane fraction (upper panel, compare lanes 3 and 4) without affecting the membrane association of Tor2 (lower panel, compare lanes 3 and 4). Consistent with the reduction in the membrane-bound Tap42, the amount of Tap42 co-immunopurified with Tor2 from the total cell extract was also diminished upon rapamycin treatment (Figure 3B, compare lanes 1 and 2). These findings suggest that the association of Tap42 with TORC1 is sensitive to rapamycin.

Figure 3 The association of Tap42 with Tor2 is sensitive to rapamycin. Exponentially growing cells expressing HA-TOR2 (BY2470) were treated with (+) or without (-) 200 nM of rapamycin for 1 h and lysed. (A) Cell extracts were partitioned into soluble and membrane fractions. The distribution of Tap42 (upper panel) and HA-Tor2 (lower panel) in the total extracts (T) and membrane fraction (P₁₀₀) was determined by immunoblotting. (B) Extracts from cells (BY2470) treated with (+) or without (-) rapamycin were treated with Triton X-100, and precipitated with anti-Tap42 antibody. The presence of Tap42 and HA-Tor2 in the precipitates was determined by immunoblotting.

Tap42 is phosphorylated only when it is associated with TORC1

Previously, we have shown that Tap42 is phosphorylated by the Tor kinases in a rapamycin-sensitive manner (Jiang and Broach, 1999). The physical association of Tap42 with TORC1 is consistent with the fact of Tap42 being direct target of the Tor kinases. As the TORC1-associated Tap42 represents a small fraction of the protein, it is possible that only this portion of Tap42 is phosphorylated by the Tor kinases. To test this notion, we radiolabeled yeast cells with ³²P and partitioned the labeled cell extract into soluble and membrane fractions. The phosphorylation state of Tap42 in each fraction was determined by autoradiography of the immunopurified protein. As shown in Figure 4A, we found that the membrane-associated Tap42 was phosphorylated, as indicated by the incorporation of radioactive ³²P into the protein. Even though the soluble fraction contained the majority of Tap42 (upper panel, lane 2), we found that Tap42 in this fraction was virtually unphosphorylated, as indicated by the absence of ³²P labeling (lower panel, lane 2). This result suggests that Tap42 is phosphorylated only when it is associated with TORC1.

We have previously shown that Tap42 undergoes dephosphorylation in cells treated with rapamycin (Jiang and Broach, 1999). In light of the current finding that Tap42 is phosphorylated only in association with TORC1, it is possible that rapamycin induces Tap42 dephosphorylation by disrupting its association with TORC1. To test this possibility, we monitored the distribution of the phosphorylated Tap42 in cells treated with rapamycin. As shown in Figure 4B, rapamycin treatment caused a rapid dissociation of Tap42 from the membrane fraction (compare lanes 1 and 2), which was accompanied by the disappearance of radiolabeled Tap42 from the membrane fraction and its simultaneous reappearance in the soluble fraction (compare lanes 5 and 6). This finding indicates that phosphorylated Tap42 is released into the cytosol from TORC1 by rapamycin. Following the release, the levels of radiolabeled Tap42 decreased gradually, suggesting that Tap42 underwent dephosphorylation in the cytosol. The rate of dephosphorylation in the cytosol is consistent with that when Tap42 dephosphorylation was monitored in the total cell extract (Jiang and Broach, 1999).

Figure 4 Tap42 is phosphorylated only when it is associated with TORC1. (A) Exponentially growing cells (Y959) were metabolically labeled with ³²P and lysed. Cell extracts were partitioned into soluble and membrane fractions. The total cell extracts (T), soluble (S₁₀₀) and the resuspended membrane (P₁₀₀) fractions were boiled in the presence of SDS, diluted with buffer containing Triton X-100 and then precipitated with anti-Tap42 antibody. (B) Exponentially growing cells (Y959) were metabolically labeled with ³²P followed by treatment with rapamycin. An aliquot of cells was removed at the indicated time point after addition of the drug and processed as described above. The precipitated Tap42 protein was detected by immunoblotting (IB) and the incorporation of the radiolabel in the protein was visualized by autoradiography (³²P).

The Tap42-phosphatase complexes associate with TORC1 in a rapamycin-sensitive manner

In yeast, Sit4 and PP2Ac are two major target phosphatases of Tap42. Their interaction with Tap42 is Tor-dependent and plays a critical role in Tor-mediated signaling (Di Como and Arndt, 1996). It is thus possible that the portion of Tap42 associated with TORC1 is in complex with Sit4 or PP2Ac. To test this notion, we first examined whether the two phosphatases were also present in the membrane fraction, and whether the membrane association was sensitive to rapamycin. The PP2Ac in yeast is encoded by two genes, *PPH21* and *PPH22*, whose products are ~90% identical to each other (Sneddon *et al*, 1990). We focused on only one of the gene products, Pph21. As shown in Figure 5A, we found that whereas most of Pph21 and Sit4 was in the soluble fraction (not shown), a small percentage of each phosphatase was in the membrane fraction (compare lanes 1 and 3). A quantitative densitometry analysis of the immunoblot revealed that approximately 10% of Pph21 and 5% of Sit4 was associated with the membrane fraction. Furthermore, we found that rapamycin treatment reduced the amount of both phosphatases in the membrane fraction to a level barely detectable (Figure 5A, compare lanes 3 and 4), suggesting that the membrane association of both Pph21 and Sit4 was rapamycin sensitive. To confirm that both Sit4 and Pph21 existed in complex with TORC1, we immunoprecipitated HA-tagged Tor2 from detergent-solubilized membrane fraction and examined the presence of the two phosphatases in the precipitate. As shown in Figure 5B, anti-HA antibody effectively precipitated Tap42, Pph21 and Sit4 from extracts expressing HA-tagged Tor2 but not from extracts expressing untagged Tor2 (compare lanes 1 and 2). This result suggests that like Tap42, Sit4 and Pph21 are associated with TORC1. In addition, as rapamycin almost completely abolished the association of Sit4 and Pph21 with the membrane fraction, it is conceivable that most, if not all, of the membrane-bound phosphatases is in complex with TORC1.

Figure 5 The Tap42-phosphatase complexes are associated with TORC1. (A) Cells expressing HA-Pph21 (Y162) were grown to early log phase and treated with (+) or without (-) 200 nM of rapamycin for 1 h. Cells were lysed and extracts were centrifuged at 100 000 g to obtain membrane fractions. The distribution of HA-Pph21 (upper panel), Sit4 (middle panel) and Tap42 (lower panel) in the total cell extracts (Total) and membrane fractions (P₁₀₀) in response to rapamycin treatment was determined by immunoblotting. (B) Wild-type cells (Y959) and cells expressing myc-Pph21 together with HA-TOR2 (Y992) were grown to early log phase and lysed. The membrane fraction of the cell extracts was solubilized with Triton X-100 and precipitated with anti-HA antibody. The presence of HA-Tor2, Tap42, myc-Pph21 and Sit4 in the precipitates was determined by immunoblotting.

mycin almost completely abolished the association of Sit4 and Pph21 with the membrane fraction, it is conceivable that most, if not all, of the membrane-bound phosphatases is in complex with TORC1.

The association of the Tap42-phosphatase complexes with TORC1 is resistant to rapamycin in tap42-11 cells

Mutations in Tap42 have been shown to render yeast cells resistant to rapamycin. One of the alleles, *tap42-11*, is both temperature sensitive (*Ts*⁻) and rapamycin resistant (Di Como and Arndt, 1996). In our previous study, we demonstrated that the *Ts*⁻ phenotype was caused by defects in the interaction of the mutant Tap42 protein with phosphatases (Wang *et al*, 2003). However, the mechanism for the rapamycin-resistant trait associated with this allele remained unclear. Upon finding that the Tap42-phosphatase complexes associates with TORC1 in a rapamycin-sensitive manner, it is thus plausible that the drug-resistant trait may be originated from an altered association of the Tap42 complexes with TORC1. To test this notion, we examined the association of Tap42 and Sit4 with the membrane fraction in the *tap42-11* mutant cells treated with rapamycin. As shown in Figure 6, the membrane association of Tap42 in the *tap42-11* cells was largely unaffected by rapamycin treatment (middle panel, compare lanes 3 and 4), which was in contrast to what occurred in the wild-type cells, in which most of the membrane-bound Tap42 disappeared (middle panel, compare lanes 1 and 2). Similarly, rapamycin failed to abrogate the association of Sit4 with the membrane fraction in the mutant cells (lower panel). This result indicates that the association of the Tap42-phosphatase complexes with TORC1 is resistant to rapamycin in the mutant cells.

The Tap42-phosphatase complexes exist mainly on membrane structures

Under optimal growth conditions, approximately 5–10% of Pph21 and Sit4 is found to be associated with Tap42 (Di Como

Figure 6 The association of the Tap42-phosphatase complexes with membrane is resistant to rapamycin in *tap42-11* cells. Wild-type (Y959) and *tap42-11* cells (Y176) were grown to early log phase and treated with (+) or without (–) 200 nM of rapamycin for 1 h. Cells were lysed and extracts were centrifuged at 100 000 g to obtain membrane fractions. The presence of Tap42 in the total cell extracts (Total) and that of Tap42 and Sit4 in the membrane fractions (P₁₀₀) in response to rapamycin treatment was determined by immunoblotting.

Figure 7 The Tap42-phosphatase complexes exist mainly on membrane. Cells expressing HA-Pph21 (Y162) were grown to early log phase and lysed. Cell lysates were partitioned into soluble and membrane fractions by centrifugation at 100 000 g. The presence of Tap42, Sit4 and HA-Pph21 in the fractions was determined by immunoblotting (IB). The total extracts (T), soluble (S₁₀₀) and membrane (P₁₀₀) fractions were treated with Triton X-100, and precipitated with anti-Tap42 antibody. The presence of Tap42, Sit4 and HA-Pph21 in the precipitates from each fraction was determined by immunoblotting (IP). The amount of Tap42 shown in the IB panel represents half of the input used for the precipitation shown in the IP panel. The amount of Sit4 and HA-Pph21 shown in the IB panel represents 10% of the input used for the precipitation shown in the IP panel.

and Arndt, 1996), which is close to the amount of the two phosphatases found in the membrane fraction (Figure 5A). This correlation raises the possibility that the Tap42-phosphatase complexes exist only in the membrane fraction. To test this possibility, we obtained the soluble and membrane fractions of yeast cell lysate, and assessed the levels of the Tap42-Sit4 and Tap42-Pph21 complexes in each fraction by determining the amount of Sit4 and Pph21 co-immunoprecipitated with Tap42. As shown in Figure 7, both Sit4 and Pph21 were effectively co-precipitated with Tap42 from the membrane fraction (lane 3). In contrast, only a trace amount of Pph21 and Sit4 was co-precipitated with Tap42 from the soluble fraction, even though most of these proteins were in the fraction (lane 2). This result indicates that the Tap42-phosphatase complexes exist mainly in association with membrane structures. Furthermore, the rapamycin-sensitive nature of the association (Figure 5A) suggests that the com-

Figure 8 Rapamycin induces rapid redistribution of the Tap42-phosphatase complexes into the cytosol. Cells of strain Y162 were grown to early log phase and treated with 200 nM of rapamycin. Aliquots of cells were removed from the culture at the indicated time points after addition of the drug, and lysed immediately. The cells lysates were partitioned into membrane and soluble fractions by centrifugation at 100 000 g. (A) The amount of Tap42, Sit4 and HA-Pph21 associated with the membrane fraction from samples taken at the indicated time points after addition of rapamycin was determined by immunoblotting. (B) The soluble fractions from samples taken at the indicated time points were immunoprecipitated with anti-Tap42 antibody, and the amount of Tap42, Sit4 and HA-Pph21 in the precipitates was determined by immunoblotting. (C) The level of Tap42, Sit4 and HA-Pph21 in the total cell extracts at the indicated time points after rapamycin treatment was determined by immunoblotting. The first lane, depicted as 'U', shows the result of the untreated cells.

plexes become membrane-bound through their connection with TORC1.

Rapamycin rapidly releases the Tap42-phosphatase complexes into the cytosol

It has been known that rapamycin induces a rapid activation of phosphatases (Schmidt *et al*, 1998; Jacinto *et al*, 2001). However, the mechanism for this rapid process remains unclear. The above finding that the Tap42-phosphatase complexes associate with TORC1 in a rapamycin-sensitive manner suggests that disruption of this association may play a role in the rapamycin-induced phosphatase activation. We thus determined the time required for rapamycin to dislodge both Tap42 and phosphatases from the membrane fraction. Accordingly, we obtained the membrane fractions from cells treated with rapamycin for various times and determined the amount of Tap42, Sit4 and Pph21 in the fractions. As shown in Figure 8A, most of Tap42, Sit4 and Pph21 was released from the membrane fraction within 10 min of the drug treatment, as indicated by the significant decrease in the amount of each protein in the fraction. Because rapamycin may continue acting on the Tap42-TORC1 association during sample preparation, one control was also included in which

cells were processed immediately after adding rapamycin. In comparison with the untreated cells, this sample contained a comparable amount of Tap42 and phosphatases in the membrane fraction (Figure 8A, compare lanes 1 and 2), suggesting that rapamycin did not significantly affect the membrane association of the Tap42–phosphatase complexes during sample preparation. Taken together, these results indicate that rapamycin induces rapid dissociation of Tap42 and phosphatases from TORC1.

Next, we determined whether Tap42 and phosphatases were released together as a complex or separately from TORC1 in response to rapamycin treatment. Accordingly, we monitored the appearance of the Tap42–Sit4 and Tap42–Pph21 complexes in the soluble fraction of the same cell samples used above by co-immunoprecipitation with anti-Tap42 antibody. As shown in Figure 8B, a trace amount of Sit4 and Pph21 was co-precipitated with Tap42 in untreated cells (lane 1). Rapamycin caused a slight increase in the levels of the phosphatases co-precipitated with Tap42 during sample preparation (compare lanes 1 and 2). The amount of Sit4 and Pph21 co-precipitated with Tap42 was drastically increased within 10 min of the drug treatment (compare lanes 2 and 3), which mirrored the significant decrease in the membrane-bound portion of the phosphatases (Figure 8A), indicating a rapid release of the Tap42–phosphatase complexes into the cytosol. Following the initial increase, the amount of Sit4 and Pph21 co-precipitated with Tap42 started reducing after 10 min of rapamycin treatment, and an extensive reduction was observed after 30 min (Figure 8B). As the levels of Sit4 and Pph21 in the total cell extract remained the same over a course of 60 min (Figure 8C), it is unlikely that the reduction was caused by degradation of both proteins. Therefore, the diminishing amount of Sit4 and Pph21 co-precipitated with Tap42 over time indicates a slow dissociation of both proteins from Tap42, suggesting that the Tap42–phosphatase complexes were disassembled following their release from TORC1. Interestingly, the rate of the disassembly showed a straight correlation with that of Tap42 dephosphorylation (Figure 4B), suggesting that the latter may be the cause for the disassembly. Taken together, the above observations indicate that rapamycin causes a rapid release of the Tap42–phosphatase complexes from TORC1. However, the disassembly of the complexes is not a direct consequence of rapamycin action and is likely to be caused by dephosphorylation of Tap42.

Nutrient starvation causes the release of the Tap42–phosphatase complexes from TORC1

Both TORC1 and the Tap42–phosphatase complexes are part of a nutrient-sensing machinery that controls cell growth in response to changes in nutrient conditions. It is thus possible that the association of the Tap42–phosphatase complexes with TORC1 is regulated by changes in nutrient availability. To test this possibility, we examined the association in response to nutrient deprivation. Accordingly, cells grown in nutrient-rich medium were shifted to water, and the association of Tap42 with Tor2 and phosphatases was examined by co-immunoprecipitation. As shown in Figure 9, we found that Tor2, Sit4 and Pph21 were co-precipitated with Tap42 when cells were grown in nutrient-rich medium (lane 5). However, upon shifting the cells to water, the amount of Tor2, Sit4 and Pph21 co-purified with Tap42 decreased over

Figure 9 Nutrient starvation causes a rapid release of the Tap42–phosphatase complexes from TORC1. Exponentially growing yeast cells (Y992) were shifted from YP medium to water. Aliquots of cells were collected at the indicated time points after the shift and lysed. The levels of Tap42, HA-Tor2, Sit4 and myc-Pph21 in the cell extracts were determined with immunoblotting (extract). Cell extracts were precipitated with Tap42 antibody. The amount of Tap42, HA-Tor2, Sit4 and myc-Pph21 in the precipitates was determined by immunoblotting (IP).

time (right panels), whereas the levels of the proteins in the total cell extracts remained unchanged (left panels), suggesting that Tor2 and the phosphatases dissociated from Tap42 in response to the shift. Importantly, the amount of Tor2 co-purified with Tap42 was significantly lower at the 10 min time point than that before the shift (compare lanes 5 and 6), whereas the amount of Sit4 and Pph21 co-purified with Tap42 remained unchanged until the 30 min time point (compare lanes 6 and 7). This finding suggested that Tor2 dissociated from Tap42 before the phosphatases, indicating that the disassembly of the Tap42–phosphatase complexes occurred after their release from TORC1. Thus, the response of the Tap42–phosphatase complexes to changes in nutrient availability is similar to that when cells were treated with rapamycin (Figure 8).

Discussion

The Tap42–phosphatase complexes are the key factors in the rapamycin-sensitive signaling pathway and are responsible for mediating gene expression in response to Tor signaling activity (Shamji *et al*, 2000; Duvel *et al*, 2003). The components in the complexes, including Tap42, PP2A and Sit4, are all soluble proteins, which are found mainly in the cytosol. It is thus surprising that the Tap42–phosphatase complexes exist mainly on membrane structures. Two lines of evidence suggest that these complexes are associated with TORC1. First, the membrane association of Tap42, Pph21 and Sit4 is rapamycin sensitive, suggesting a Tor-dependent process (Figure 5). Second, Tap42 is co-immunopurified with components of TORC1, including Tor1, Tor2 and Kog1 (Figure 2). Because TORC1 associates with membrane structures (Kunz *et al*, 2000; Wedaman *et al*, 2003), it is conceivable that the association with TORC1 is what allows the Tap42 complexes to become membrane-bound. It is worthy noting, however, that not all the membrane-bound Tap42 is sensitive to rapamycin. We have consistently observed a small fraction of Tap42 that remains in the membrane fraction upon rapamycin treatment (Figures 3–6). It is possible that this portion of Tap42 may associate with membrane structures independent of TORC1.

Tap42 and its associated phosphatases were not found as the components of TORC1 when the complex was first isolated. This is not surprising given the fact that the initial components of TORC1 were identified as major species of proteins appearing in SDS-PAGE that were co-purified with either Tor1 or Tor2. The amount of Tap42 and its associated phosphatases might be low and thus escaped the detection, or their signals on SDS-PAGE were masked by nonspecific proteins (Loewith *et al*, 2002; Wedaman *et al*, 2003). The identification of another component of TORC1, Tco89, upon further characterization of TORC1, is an indication that this complex contains additional components (Reinke *et al*, 2004). It is plausible that TORC1 may represent a stable core complex, whose activity is regulated through its associations with many peripheral proteins that act either as regulators or effectors of the core complex. As Tap42 is a direct target of Tor, its association with TORC1 is in accordance with the notion.

The portion of Tap42 that is associated with TORC1 accounts for only 10% of the protein. Interestingly, it is this portion of Tap42 that is phosphorylated and the majority of Tap42, which resides in the cytosol, is not (Figure 4). This observation suggests that a physical association with TORC1 is prerequisite for Tor-dependent phosphorylation of Tap42. On the other hand, Tap42 is found in complex with phosphatases only when it is associated with TORC1 (Figure 7), suggesting that the Tor-dependent phosphorylation of Tap42 is required for its interaction with phosphatases. Taken together, it is plausible that a physical association between Tap42 and TORC1 applies a spatial restriction so that only a small portion of Tap42 is phosphorylated, which in turn is able to accommodate the binding of a limited amount of phosphatases. This theme explains how Tap42 is able to selectively interact with a small set of Sit4 and PP2Ac.

How does Tap42 interact with TORC1? As mentioned in the introduction, TORC1 contains two essential components, Lst8 and Kog1, in addition to Tor1 or Tor2 (Loewith *et al*, 2002). Tap42 differs from these two components in that the association of Tap42 with TORC1 is rapamycin sensitive, whereas the association of the two with Tor is not. It is thus unlikely that these two components serve as adaptors to bridge the association of Tap42 with Tor. One possibility is that Tap42 may interact with Tor through an unidentified adaptor protein, whose association with Tor is rapamycin sensitive. A counterpart of this putative protein has been identified in mammalian cells. The adaptor protein, raptor, whose interaction with mTOR is sensitive to rapamycin, acts in targeting mTOR to downstream substrates (Hara *et al*, 2002; Kim *et al*, 2002; Nojima *et al*, 2003). Alternatively, Tap42 may bind directly to Tor in such a way that the binding of rapamycin-FKBP dimer to Tor is able to dislodge Tap42 from TORC1. Future study will be aimed to distinguish these two possibilities, which will provide insight regarding how the Tor kinases exert their signaling activity.

Previous studies have shown that rapamycin induces a rapid activation of the Tap42-associated phosphatases as well as their dissociation from Tap42 (Schmidt *et al*, 1998; Beck and Hall, 1999; Cardenas *et al*, 1999). It has thus been suggested that Tap42 negatively regulates phosphatase activity and that dissociation of Tap42 from phosphatases by the action of rapamycin results in phosphatase activation. According to this model, the speed by which the Tap42-

associated phosphatases are activated depends on the rate of their dissociation from Tap42, which is a consequence of inhibition of Tor kinase activity by rapamycin. However, there is no evidence showing that the phosphatases dissociate from Tap42 at a rate that matches that of phosphatase activation induced by rapamycin. On the other hand, an increasing body of evidence has suggested that Tap42 may play a positive role in phosphatase regulation. We have previously shown that Tap42 was required for the activity of the phosphatases to which it associates (Wang *et al*, 2003). Similarly, Duvel *et al* (2003) have recently demonstrated that inactivation of Tap42 severely attenuated the rapamycin-induced expression levels of the genes in the nitrogen discrimination pathway, a process that is dependent on the activity of Sit4. Furthermore, an earlier study has shown that when Tap42 was co-overexpressed together with either Pph21 or Sit4, it created a growth inhibitory activity, which was more pronounced than it was when the phosphatases were expressed alone (Di Como and Arndt, 1996). A negative role of Tap42 in phosphatase regulation cannot accommodate all these observations.

The rapid dissociation of the Tap42-phosphatase complexes from TORC1 in response to nutrient starvation or rapamycin treatment indicates that regulating the association is a critical step in Tor-mediated signaling. The timing of the release induced by rapamycin correlates with that of the phosphatase activation previously reported (Schmidt *et al*, 1998; Beck and Hall, 1999; Cardenas *et al*, 1999; Hardwick *et al*, 1999). On the other hand, the dissociation of phosphatases from Tap42, either upon nutrient starvation or rapamycin treatment, takes place at a slower rate. In the case of rapamycin treatment, most of the phosphatases remained bound with Tap42 after 30 min of the drug treatment (Figure 8), which cannot account for the rapid phosphatase activation that occurs within a few minutes of the drug treatment. It is likely that the release of the Tap42-phosphatase complexes from TORC1 causes the activation of the Tap42-associated phosphatases. In support of this view, it has been shown that rapamycin failed to induce phosphatase activation in the *tap42-11* cells (Schmidt *et al*, 1998), in which the association of the Tap42-phosphatase complexes with TORC1 is resistant to rapamycin (Figure 6).

Interestingly, the disassembly of the Tap42-phosphatase complexes following its release into the cytosol occurs at a rate similar to that of Tap42 dephosphorylation (Jiang and Broach, 1999). This correlation suggests that the disassembly may be a result of Tap42 dephosphorylation, which is an indirect consequence of rapamycin action that prevents Tap42 from accessing the Tor kinases. In this regard, rapamycin acts not by directly inhibiting the kinase activity of Tor but by disrupting its association with the targets. How long the Tap42 complexes can last upon release from TORC1 may depend on the activity of the phosphatase that dephosphorylates Tap42, which we have previously shown to be the PP2A holoenzyme (Jiang and Broach, 1999).

Taken together, our findings promote the model shown in Figure 10. In actively growing cells, the Tap42-phosphatase complexes are tethered to membrane structures through their association with TORC1. Nutrient starvation or rapamycin treatment dislodges the complexes from TORC1 and releases them into the cytosol, where the Tap42-associated phosphatases become active. Disassembly of the Tap42 complexes

occurs when Tap42 loses its phosphorylation, owing to the action of the PP2A holoenzyme in the cytosol, and this process terminates the phosphatase activity (Figure 10).

There are two important implications in this model that challenge the current view regarding the role of Tap42 in phosphatase regulation. First, this model predicts that Tap42 is a positive regulator of the phosphatases to which it associates. This positive role is supported by the substantial evidence mentioned above. Second, it predicts that the rapamycin-induced phosphatase activation is a transient process, which terminates upon disassembly of the Tap42–phosphatase complexes. The second prediction, albeit surprising, is a logical deduction from the first one. Circumstantial indications do exist that rapamycin action is a transient process in transcriptional regulation, to which Tap42 is a central regulator. Analysis of 297 genes whose expression level changed more than four-fold in response to rapamycin revealed a remarkable yet unexplained finding that the changes in the expression level were brief, which peaked around 15–30 min after the drug treatment and relaxed afterwards (Hardwick *et al*, 1999). The timing of the relaxation correlates with that of the disassembly of the Tap42–phosphatase complexes (Figure 8B). Further study will determine whether there is a causal connection between these two events. Altogether, our model accounts for many previous findings that are contradictory to a negative role of Tap42 in phosphatase regulation. Although this model needs to be further tested, it does provide an effective explanation for the action of rapamycin in regulating the Tap42–phosphatase complexes, and likely, other targets of the Tor kinases.

Figure 10 Model for rapamycin-induced phosphatase activation. The Tap42–phosphatase complexes exist mainly on membrane structures through association with TORC1. Rapamycin abrogates the association and releases the complexes into the cytosol, where they become active. Disassembly of the Tap42 complexes occurs when Tap42 is dephosphorylated by the PP2A holoenzyme in the cytosol, which terminates the Tap42-dependent phosphatase activity. Pase: phosphatase.

Materials and methods

Yeast strains and reagents

Yeast strains used in this study are listed in Table I. All strains are derivatives of strain W303 (Thomas and Rothstein, 1989). Yeast cells were normally grown in YP or synthetic complete (SC) medium lacking appropriate amino acid(s) for plasmid selection. All media contained 2% glucose as the carbon source. For the ^{32}P -labeling experiment, yeast cells were grown in low-phosphate YP medium containing 2% glucose (Rubin, 1973). Standard methods were used for yeast transformation and other manipulations (Guthrie and Fink, 1991). Rapamycin was purchased from LC Laboratories (MA) and was stored in 10% Tween 20 and 90% ethanol at a concentration of 1 mM and was added to growth medium to a final concentration of 200 nM. Protease inhibitor cocktails were from Roche Applied Science. Anti-Tap42 and -Sit4 antibodies have been described previously (Wang and Jiang, 2003). Anti-Tap42 and -HA antibodies were crosslinked to Protein A beads as described (Wang *et al*, 2003). Anti-myc (9E10) and anti-HA (12CA5) antibodies were purchased from Roche Applied Science.

Plasmid and strain construction

Epitope tagging of PPH21: Y992 was created by replacing the endogenous PPH21 gene in BY2470 with a triple myc epitope-tagged PPH21 gene using two-step gene replacement (Rothstein, 1991). The plasmid containing a 5' end triple myc epitope-tagged PPH21 gene (pRS406 (URA3)-(myc)₃PPH21) was a kind gift from Dr Broach at Princeton University. This plasmid was linearized by digesting with BglII at the site inside the PPH21 gene and transformed into BY2470 for integration at the PPH21 locus. The resulting strain was subsequently placed on 5-fluoroorotic acid (5-FOA) plate to deselect the URA3 gene. 5-FOA-resistant colonies were then screened for those expressing myc-tagged PPH21 (Y992) by immunoblotting.

Epitope tagging of Kog1 and Avo1: A DNA fragment corresponding to the region that spanned the stop codon (−417 to +236) of the KOG1 gene was PCR amplified from yeast genomic DNA and cloned into pRS406 (URA3) using KpnI and XhoI restriction sites. A BamHI site was then introduced immediately before the stop codon by site-directed mutagenesis. A DNA fragment containing triple tandem repeats of HA epitope sequence was PCR amplified and cloned into the BamHI site that was so engineered that the HA epitope sequence was fused in-frame with the coding region of KOG1. The resulting plasmid, pRS406-(HA)₃ kog1, was linearized by digestion with BglII, and transformed into Y959 to replace the wild-type KOG1 gene with the HA-tagged version by two-step gene replacement as described above. The strain carrying HA-tagged KOG1 (Y1032) grew at a rate indistinguishable from that of wild-type strain (not shown), suggesting that the tagged gene was fully functional. Y1031, a strain expressing a triple HA-tagged AVO1 gene, was generated in a similar way. A PCR-amplified DNA fragment containing the 3' end portion of AVO1 (−781 to +792 to the stop codon) was cloned into pRS406 and a triple HA epitope sequence was inserted at the second last codon of the gene. The resulting plasmid, pRS406-(HA)₃ avo1, was linearized by digestion with EcoRI and transformed into Y959 for gene replacement. The HA-tagged AVO1 was found to be fully functional in support of cell growth (not shown).

Table I Strains used in this study

Strains	Genotype ^a	Source
BY2470	<i>MAT a</i> <i>tor2::LEU2 tor1::HIS3</i> [pRS314 (<i>CEN TRP1</i>)-(HA) ₃ TOR2]	Jiang and Broach (1999)
Y162	<i>MAT α</i> <i>pph21::(HA)₃PPH21</i>	Wang and Jiang (2003)
Y176	<i>MAT α</i> <i>tap42::HIS3</i> [pRS415 (<i>CEN LEU2</i>)- <i>tap42-11</i>]	Wang and Jiang (2003)
Y959	<i>MAT α</i> <i>W.T.</i> (W303-1B)	Lab stock
Y990	<i>MAT a</i> <i>tor1::HIS3</i> [pRS314 (<i>CEN TRP1</i>)-(HA) ₃ TOR1]	This study
Y992	<i>MAT α</i> <i>pph21::(myc)₃PPH21 tor2::LEU2 tor1::HIS3</i> [pRS314 (<i>CEN TRP1</i>)-(HA) ₃ TOR2]	This study
Y1031	<i>MAT α</i> <i>avo1::(HA)₃AVO1</i>	This study
Y1032	<i>MAT α</i> <i>kog1::(HA)₃KOG1</i>	This study

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

^aPlasmids are indicated in square brackets. Yeast markers carried on each plasmid are listed in parentheses following the plasmid designation.

Preparation of cell lysates and fractionation

Exponentially growing cells were harvested, washed $2 \times$ with ice-cold 10 mM sodium azide and converted to spheroplasts as described before (Jiang and Broach, 1999). Spheroplasts were lysed with lysis buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 50 mM NaF, 50 mM sodium pyrophosphate and protease inhibitor cocktails. Unbroken cells were removed by centrifugation at 500 g for 5 min at 4°C. To obtain soluble and membrane fractions, cell lysates (Total) were subjected to centrifugation at 100 000 g for 45 min at 4°C. The supernatant was designated as soluble fraction (S_{100}) and pellet as membrane fraction (P_{100}). For experiments designed to determine the release of Tap42-phosphatase complexes in response to rapamycin treatment or nutrient starvation (Figures 8 and 9), cells were lysed with glass beads. Briefly, at the indicated time points after the treatment, aliquots of cells were removed and immediately mixed with the same volume of ice in a centrifuge tube before being collected by centrifugation. Cells were washed once with ice-cold lysis buffer, and lysed immediately with glass beads. Beads and unbroken cells were removed by centrifugation at 500 g for 5 min at 4°C. Lysates were either partitioned into membrane and soluble fractions as described above, or used directly for immunoprecipitation.

Co-immunoprecipitation

For co-immunoprecipitation with cell lysates or soluble fractions, samples were incubated with 0.5% of Triton X-100 on ice for 30 min before adding antibodies. For co-immunoprecipitation with membrane fractions, the 100 000 g pellet was resuspended in lysis buffer half volume of its corresponding supernatant. Triton X-100 was added to the suspension to a final concentration of 1%. After incubation on ice for 30 min, the suspension was diluted two-fold with lysis buffer and used for co-immunoprecipitation as described before (Wang *et al*, 2003).

³²P-radiolabeling, fractionation and immunoprecipitation

Yeast cells were grown to early log phase in low-phosphate YP medium (LPM) for 24 h and transferred to fresh LPM for an additional 3 h. For the experiment shown in Figure 4A, cells (30 OD₆₀₀ units) were harvested and resuspended in 10 ml of LPM

containing 5 mCi of ³²PO₄ (Perkin-Elmer). After 3 h labeling, cells were harvested, washed once with ice-cold 10 mM sodium azide and lysed with glass beads in 1 ml of lysis buffer containing protease and phosphatase inhibitors as described above, and unbroken cells were removed by centrifugation at 500 g for 3 min. Cell lysate was split and one half (0.5 ml) was partitioned into soluble and membrane fractions by centrifugation at 100 000 g. For the experiment shown in Figure 4B, cells (100 OD₆₀₀ units) were labeled with 15 mCi of ³²PO₄ for 3 h. Rapamycin was then added to the culture in the presence of ³²PO₄ at a final concentration of 200 nM. An aliquot of cells (~30 OD₆₀₀ units) was removed at the indicated time points after addition of the drug and lysed. Cell lysates were fractionated into membrane and soluble fractions. The membrane fraction was resuspended in lysis buffer with a volume equal to that of the soluble fraction. All fractions were added with SDS to a final concentration of 0.5%, and boiled for 5 min followed by a four-fold dilution with lysis buffer containing 0.5% Triton X-100. After removal of insoluble cell debris by centrifugation at 12 000 g for 5 min, each sample was incubated with 20 µl of anti-Tap42 antibody-conjugated Protein A beads for 3 h. Beads were washed $4 \times$ with Tris buffer, pH 7.4, containing 300 mM NaCl and 1% Triton X-100, and $2 \times$ with 1% β-mercaptoethanol. After the final wash, beads were resuspended in 70 µl of $2 \times$ SDS sample buffer and boiled for 5 min. Proteins were separated by electrophoresis on a 10% polyacrylamide SDS gel and transferred to nitrocellulose membrane. The presence of Tap42 in the membrane was detected using anti-Tap42 antibody in conjunction with ECL detection reagents (Amersham). The membrane was left overnight to allow the chemofluorescent signal fade. Radiolabeled Tap42 was then visualized by autoradiography.

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