HEAT Repeats Mediate Plasma Membrane Localization of Tor2p in Yeast*

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The subcellular distribution of Tor1p and Tor2p, two phosphatidylinositol kinase homologs and targets of the immunosuppressive drug rapamycin in Saccharomyces cerevisiae, was analyzed. We found that Tor protein is peripherally associated with membranes. Subcellular fractionation and immunofluorescence studies showed that Tor1p and Tor2p associate with the plasma membrane and a second fraction that is distinct from Golgi, vacuoles, mitochondria, and nucleus and may represent vesicular structures. Pulse-chase experiments showed that association of Tor protein with plasma membrane and the second compartment is fast, does not appear to involve components of endocytic, secretory, or Golgi to vacuole transport pathways, and is not affected by the immunosuppressive drug rapamycin. Deletion analysis reveals that two domains within Tor2p independently mediate localization to both compartments. These domains are composed of HEAT repeats that are thought to act as protein-protein interaction surfaces. Our studies therefore place Tor proteins at the site of action of their known downstream effectors and suggest that they may be part of a multiprotein complex.

The Saccharomyces cerevisiae Tor1p and Tor2p proteins are components of a novel signaling pathway that controls cell growth in response to nutrient availability (1, 2). Tor1p and Tor2p were originally identified by dominant mutations that confer resistance to the immunosuppressive drug rapamycin, and were later shown to be the physical targets of the FKBP12-rapamycin complex (3–9). Tor1p and Tor2p are highly homologous and members of the phosphatidylinositol kinase-related kinase family (10). Phosphatidylinositol kinase-related kinases despite their homology to phosphatidylinositol 3- and 4-kinases are thought to function as protein kinases (11).

Tor2p is an essential protein that regulates cell growth in two ways. First, it acts redundantly with Tor1p in a nutrient-sensing signaling cascade that is required for translation initiation and G1 progression. This function is inhibited by rapamycin (1, 2). Second, Tor2p is also required for cell cycle-dependent reorganization of the actin cytoskeleton (12, 13). This function is unique to Tor2p, is also essential, and is not sensitive to rapamycin. Accordingly, yeast cells lacking Tor1p and Tor2p activity or cells treated with rapamycin exhibit a dramatic reduction in translation initiation, and show all characteristics of nutrient-starved cells, including arrest in the early G1 phase of the cell cycle, accumulation of the storage carbohydrate glycogen, degradation of amino acid transporters, transcriptional and morphological changes, down-regulation of ribosome biogenesis, and autophagy (14–20). Loss of Tor2p function alone, on the other hand, leads to arrest throughout the cell cycle and depolarization of the actin cytoskeleton (12, 13, 21).

The Tor signaling pathway appears to be evolutionarily conserved. The mammalian homolog mTOR, also known as FRAP/RAPT1/RAPT1, is highly related and acts in a rapamycin-sensitive signaling pathway that modulates translation initiation in response to mitogens or amino acid availability (2, 22–25). mTOR regulates translation by activating 70-kDa S6 kinase and inducing phosphorylation of the translational repressor 4E-BP1/PHAS-1 which, in turn, leads to dissociation of 4E-BP from eIF-4E and initiation of translation (1, 2, 26). So far, no direct role of mTOR in the regulation of actin dynamics has been demonstrated.

The mechanisms by which Tor coordinately regulates diverse cellular responses to nutrient availability are not precisely understood. Tor kinase activity controls the association of the phosphatase-associated factor Tap42p with type 2A and type 2A-related phosphatases (27–29). Tap42-mediated inhibition of phosphatase activity in turn prevents nuclear accumulation of nutrient-regulated transcription factors, inhibits degradation of amino acid transporters, and positively regulates translation initiation by modulating the phosphorylation status of downstream effectors (16–19). Tor2p, in addition, regulates actin reorganization via activation of Rho2p, a GTP exchange factor for the small GTPases Rho1p and Rho2p (12, 13). Furthermore, Ms4p, a phosphatidylinositol 4-phosphate 5-kinase, and Pcl1p, the yeast homolog of phospholipase C, have been implicated in the Tor signaling pathway suggesting a role for phosphoinositides and their metabolites in Tor function (21, 30). However, precisely how Tor1p and Tor2p are activated is not known, and no immediate downstream effectors that execute the Tor2p actin cytoskeleton function have been identified.

To gain a better understanding of Tor function, we examined the subcellular distribution of Tor1p and Tor2p and characterized domains within Tor2p that mediate localization. Here we show that Tor1p and Tor2p are peripheral membrane proteins that associate with the plasma membrane and a second compartment of high buoyant density. Tor localization to both

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† The abbreviations used are: Tor1, Tor2, target of rapamycin 1 and 2; HEAT, huntingtin, elongation factor 3, A subunit of phosphatase 2A, Tor1; FKBP12, FK506-binding protein; mTOR, mammalian target of rapamycin; 4E-BP1, eIF4E-binding protein 1; PAGEx, polyacrylamide gel electrophoresis; HA, hemagglutinin; FITC, fluorescein isothiocyanate; kb, kilobase pairs; PIPES, 1,4-piperazinediethanesulfonic acid; DAPI, 4,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; PM, plasma membrane; ConA, concanavalin A.
Strains, Plasmids, Media, and Reagents—The complete genotypes of yeast strains and a description of the plasmids used in this study are listed in Table I. Yeast strains were grown at 30 °C unless otherwise indicated. The composition of rich (YP media) or synthetic minimal media (SD) supplemented with the appropriate nutrients was as described (31). Rapamycin (kindly provided by Sandoz Pharma, Basel) was kept as a stock solution (1 mg/ml in ethanol) at −80 °C and was added to the medium to a final concentration of 0.2 μg/ml. Canavanine A (fraction IV), aprotinin, chymostatin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride were obtained from Sigma. Recombinant lytic enzyme was a gift from H. Riezman (Biozentrum, Basel). Zymolyase 20T was purchased from ICN Immunobiologicals (Irvine, CA). Protein A-Sepharose was from Amersham Pharmacia Biotech. Easytag EXPRE™S2S™ protein labeling mix was purchased from PerkinElmer Life Sciences. Mouse monoclonal antibody HA.11 or rabbit polyclonal antibodies directed against the HA tag were purchased from Berkeley Antibody Co. (Berkeley, CA). FITC-conjugated goat anti-rabbit IgG, Texas Red-labeled streptavidin, and mouse monoclonal anti-Vph1 and anti-alkaline phosphatase antisera were purchased from Molecular Probes (Eugene, OR). Rabbit anti-Cpy1p antisera was kindly provided by S. Emr (University of California, San Diego); rabbit anti-Pma1p antisera was from S. Gasser (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland); rabbit anti-Emplp7 was provided by S. Schröder (Biozentrum, Basel, Switzerland); rabbit anti-Wbp1p antisera was obtained from M. Aebi (ETH, Zurich, Switzerland); rabbit anti-hexokinase and anti-porin antisera were kindly provided by G. Schatz (Biozentrum, Basel, Switzerland).

Preparation of Polyclonal Antiserum Directed against Tor1p and Tor2p—Polyclonal antiserum against the C terminus of Tor1p and Tor2p were raised against Escherichia coli-produced His6-tagged fusion proteins containing amino acids 1680–2470 of Tor1p and amino acids 1685–2474 of Tor2p, respectively. To generate anti-peptide Tor2p antisemur, a synthetic peptide containing amino acids 16–29 of Tor2p was coupled to keyhole limpet hemocyanin by N-iodobenzoyl-N-hydrosuccinimide ester cross-linking. The resulting peptide/keyhole limpet hemocyanin conjugate or recombinant His6-Tor1p was used as the immunogen. Complete Freud’s adjuvant was repeated at least five times, once every 4 weeks. The resulting antisera were tested and titered by Western blot and immunoprecipitation experiments of yeast cell extracts.

Cell Labeling and Biochemical Extraction of Tor—Unless otherwise indicated, yeast cells were grown at 30 °C in SD medium supplemented with the appropriate amino acids and lacking methionine (SD-Met) to
an absorbance at 600 nm (A_{600}). Typically, 10 A_{600} units of cells were collected by centrifugation and resuspended in 1 ml of SD-Met medium. 120 μCi of Easytag EXPRE³S³ protein labeling mix were added, and cells were incubated for 20 min at 30 °C. A chase period was initiated by the addition of pre-warmed YPD medium (to A_{600} ≈ 0.5), and the culture was incubated for 10 min at 30 °C. The chase was terminated by addition of NaNO₃ and NaF to a final concentration of 10 mM each. Cells were centrifuged and washed once in 10 mM NaNO₃, 10 mM NaF. Cell pellets were then resuspended in 15 ml of spheroplast I buffer (25 mM AMP-PIPES, pH 6.8, 0.6 mM sorbitol, 140 mM cysteine HCl, 5 mM EDTA, 10 mM NaN₃, 10 mM NaF), incubated at room temperature for 10 min, collected by centrifugation, and resuspended to spheroplasts by lyticase digestion in spheroplast II buffer (25 mM AMP-PIPES, pH 6.8, 1.2 mM sorbitol, 140 mM cysteine HCl, 5 mM EDTA, 10 mM NaNO₃, 10 mM NaF) during a 60-min incubation at 30 °C. Spheroplasts were harvested by centrifugation at 500 × g for 4 °C, resuspended in ice-cold lysing buffer (0.5 mM EDTA, 1 mM dithiothreitol, 10 mM NaN₃, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride), and homogenized by pulling 10 times through a 23-gauge syringe. Unbroken cells were removed by centrifugation at 500 × g for 4 °C for 10 min. To determine the nature of association of Tor proteins with membranes, 100,000 × g for 10 min at 4 °C and at 100,000 g described above and subjected to sequential centrifugations at 13,000 × g for 10 min, collected by centrifugation, and resuspended in 10 mM NaN₃, 10 mM NaF supplemented with cycloheximide (10 μg/ml) to inhibit new protein synthesis, and kept on ice. Cells from the 5-min time point were resuspended in 10 mM NaN₃, 10 mM NaF containing cycloheximide (10 μg/ml) and kept on ice. Cells from each time point were then converted to spheroplasts, coated with ConA in the presence of cycloheximide, washed, and resuspended in ice-cold lysis buffer essentially as described (32). Extracts were homogenized by pulling 10 times through a 23-gauge needle. The low speed plasma membrane (PM) fraction, and the high speed P100 and S100 fractions were obtained by sequential centrifugation as described (32). The extent of cell lysis was determined by analyzing the percent of the cytosolic protein hexokinase that was recovered in the PM and P100 fractions. The amount of radioactivity in the PM, P100, and S100 fractions was expressed as percent of total radioactivity in the lysate after correction for cell lysis.

### Immunoprecipitations, SDS-PAGE, and Western Blot Analysis—P13, P100, and PM fractions were directly resuspended in immunoprecipitation boiling buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS), whereas proteins present in the S100 supernatant fractions or in sucrose gradient fractions were first precipitated with trichloroacetic acid at a final concentration of 6%. Proteins were denatured by heating to 65 °C for 10 min and subjected to immunoprecipitation in 10 ml of TNET (1% Triton X-100, 100 mM Tris-Cl, pH 8.0, 100 mM NaCl, 5 mM EDTA). Fractions were first preincubated by incubation with Sepharose CL-4B (Sigma), and then antiserum and protein A-Sepharose CL4B were added, and immunoprecipitations were incubated at 4 °C for 6 h to overnight with shaking. Tor1p and Tor2p were immunoprecipitated using the anti-Tor1p C-terminal antiserum and the anti-peptide Tor2p antiserum, respectively, except for the analysis of N-terminally truncated Tor2p mutants. In this case, the anti-Tor2p C-terminal antiserum was used. Antisera other than the anti-Tor1p or anti-Tor2p antisera were used according to personal recommendations to ensure quantitative immunoprecipitation of a given antigen. Immune complexes were recovered by centrifugation and washed five times with TNET before SDS-PAGE sample buffer was added. Samples were heated to 65 °C for 5 min to dissociate the immune complex. Immunoprecipitates were resolved by SDS-PAGE and analyzed and quantitated on a PhosphorImager.

### Results

#### Detection of Tor1p and Tor2p—To aid in the molecular analysis of Tor function, we generated antibodies that specifically recognize Tor1p or Tor2p. Tor1p-specific antibodies were raised against the C-terminal kinase domain of Tor1p, whereas the antisera against Tor2p was raised against a synthetic peptide corresponding to a unique N-terminal sequence of Tor2p. The anti-Tor1p antiserum immunoprecipitated a single polypeptide with an apparent molecular mass of 250 kDa (predicted size, 284 kDa) from 35S-labeled wild-type extracts (Fig. 1A, lane 1). The same protein was also isolated from extracts derived from cells harboring a tor2 deletion (Fig. 1A, lane 3) but not from extracts generated from cells that carried a tor1 deletion mutation (Fig. 1A, lane 2). When TOR1 was overexpressed under the control of the GAL1 promoter, up to 5-fold higher levels of the 250-kDa species were recovered (data not shown), whereas no protein was immunoprecipitated with preimmune serum (data not shown).

The anti-Tor2p antiserum specifically immunoprecipitated a single protein that migrated at approximately 250 kDa (predicted size, 285 kDa) from 35S-labeled wild-type cells (Fig. 1B, lane 1). This protein was also immunoprecipitated from an extract derived from a tor1 deletion strain (Fig. 1B, lane 2) but not from an extract generated from a tor2 mutant strain (Fig. 1B, lane 3). A 170-kDa polypeptide was immunoprecipitated from this strain instead, which corresponds in its mass to...
the predicted truncated Tor2p protein encompassing the N terminus up to the insertion point of the ADE2 disruption marker (Fig. 1B, lane 3). Overexpression of TOR2 on a multicopy plasmid led to the recovery of up to 10-fold stronger signal (data not shown). The 250-kDa species was, however, not recovered when preimmune serum was used or when excess peptide against which the anti-Tor2p antibodies were raised was present during immunoprecipitation (data not shown). Taken together, these results demonstrate that the anti-Tor1p and anti-Tor2p antisera specifically recognize endogenous Tor1p and Tor2p, respectively.

**Tor1p and Tor2p Fractionate as Peripheral Membrane Proteins**—As a first step to localize Tor1p and Tor2p, a 35S-labeled extract derived from wild-type cells was fractionated into 100,000 × g cytosolic supernatant (S100) and membrane-enriched particulate (P100) fractions. These fractions were probed by immunoprecipitation for the presence of Tor1p and Tor2p. We found that Tor1p and Tor2p mainly partition into the particulate fraction (Fig. 2 and Table II) with only minor amounts (5–10% of either Tor1p or Tor2p) detected in the soluble fraction. Overexpression of Tor1p or Tor2p led to the recovery of higher levels of Tor proteins in the soluble fraction (20–30% in the Tor overproducing cells (Table II) compared with 5–10% in wild-type cells) which could indicate that association of Tor with the particulate fraction is saturable.

To determine the nature of the association of Tor protein with the particulate fraction, a 35S-labeled extract was divided into aliquots and treated with a set of reagents prior to centrifugation. As expected, Pma1p was extracted into the S100 fraction only by treatment with the nonionic detergent Triton X-100, but not by reagents that disrupt protein-protein interaction of Tor1p or Tor2p (data not shown). The 250-kDa species was, however, not recovered when preimmune serum was used or when excess peptide against which the anti-Tor2p antibodies were raised was present during immunoprecipitation (data not shown). Taken together, these results demonstrate that the anti-Tor1p and anti-Tor2p antisera specifically recognize endogenous Tor1p and Tor2p, respectively.

**Fig. 2. Tor1p and Tor2p fractionate as peripheral membrane proteins.** A cell extract prepared from 60 A600 of 35S-labeled wild-type yeast cells was divided into aliquots and treated with the indicated reagent for 10 min on ice as described under “Experimental Procedures.” Extracts were then separated into 100,000 × g supernatant and pellet fractions, and Tor1p and Tor2p were immunoprecipitated from these fractions, resolved by SDS-PAGE, and analyzed on the PhosphorImager. S and P denote the 100,000 × g supernatant and pellet fractions, respectively. The position and molecular weights (×10^5) of prestained standards are indicated at the left. TX-100, Triton X-100.

**Second Subcellular Compartment—Subcellular fractionation studies were carried out to identify more precisely the intracellular location of Tor1p and Tor2p.** Wild-type cells were pulse-labeled with [35S]Met/Cys, converted to spheroplasts, osmotically lysed, and unbroken cells were removed by low speed centrifugation. The cell-free extract was then sequentially centrifuged at 13,000 and 100,000 × g to generate the membrane-enriched particulate P13 and P100 fractions, as well as the cytosolic S100 supernatant fraction. The presence of Tor1p and Tor2p, as well as a set of organelle marker proteins was determined in each fraction by immunoprecipitation.

The P13 fraction is highly enriched for plasma membrane, endoplasmic reticulum (ER), vacuoles, and mitochondria, whereas the P100 fraction is highly enriched for Golgi, endosomes, and secretory vesicles (Table III [34]). We found that almost all of the plasma membrane marker Pma1p, the ER membrane marker Wbp1p, the vacuolar membrane marker Vph1, and the mitochondrial outer membrane marker porin were present in the P13 fraction (Table III). The Golgi membrane marker Emp47p, in contrast, was almost exclusively found in the P100 fraction (Table III). The portion of Emp47p in the P13 fraction represents the ER-localized precursor form that is typically detected under the growth conditions used for these experiments (35). Tor1p or Tor2p were found to distribute equally between the P13 and P100 pellet fractions with only trace amounts present in the soluble fraction (Table III). The fractionation behavior of Tor1p and Tor2p is therefore different from that observed for specific organelle markers and could indicate that Tor proteins are present in more than one compartment.

To identify the subcellular membranous compartment(s) with which Tor1p and Tor2p associate, P13 and P100 fractions were resuspended in 60% sucrose and subfractionated by equilibrium flotation in continuous 18–54% sucrose density gradients. The gradients were fractionated and analyzed for the distribution of Tor1p, Tor2p, and various organelle markers. Tor1p and Tor2p present in the P13 fraction cofractionated with both each other and the plasma membrane marker Pma1p (Fig. 3). Both Tor proteins clearly fractionated in a manner distinct from vacuolar (Cyp1p) and mitochondrial (porin) markers and were also partially resolved from the ER membrane marker Wbp1p (Fig. 3A and data not shown). In the P100 fraction, Tor1p and Tor2p were present in fractions that were clearly distinct from fractions containing the Golgi marker Emp47p (Fig. 3B). Furthermore, both Tor proteins were again at least partially resolved from the ER marker Wbp1p (Fig. 3B). Similar results were obtained when yeast extracts were resolved by velocity gradient centrifugation (data not shown). In summary, Tor1p and Tor2p appear to colocalize to two biochemically distinct subcellular compartments one of which
Wild-type cells were 35S-labeled for 20 min, chased for 30 min at 30 °C and lysed, and the cell lysate was divided into equal aliquots and treated with the indicated reagents as described under “Experimental Procedures.” After treatment, extracts were separated into S100 and P100 fractions, and the amounts of Tor proteins and Pma1p in each fraction were determined by quantitative immunoprecipitation and PhosphorImager analysis. Numbers indicate percent of total.

### Table II

<table>
<thead>
<tr>
<th>Marker protein</th>
<th>P13 %</th>
<th>P100 %</th>
<th>S100 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tor1p</td>
<td>55</td>
<td>42</td>
<td>3</td>
</tr>
<tr>
<td>Tor2p</td>
<td>53</td>
<td>43</td>
<td>4</td>
</tr>
<tr>
<td>Pma1p (plasma membrane)</td>
<td>86</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Wbp1p (endoplasmic reticulum)</td>
<td>78</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Emp47p (Golgi)</td>
<td>8</td>
<td>90</td>
<td>2</td>
</tr>
<tr>
<td>Vph1p (vacuolar membrane)</td>
<td>95</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Cpy1p (vacuolar lumen)</td>
<td>48</td>
<td>6</td>
<td>46</td>
</tr>
<tr>
<td>Porin (mitochondria)</td>
<td>97</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Hexokinase (cytoplasm)</td>
<td>2</td>
<td>0</td>
<td>98</td>
</tr>
</tbody>
</table>

S, supernatant; P, pellet.

### Table III

| No treatment, endogenous | 6 | 94 |
| No treatment, GAL1 promoter | 26 | 74 |
| High salt (1 M NaCl) | 91 | 9 |
| pH 11 (0.2 M NaCl) | 86 | 14 |
| Urea (5 M) | 97 | 3 |
| Triton X-100 (1%) | 46 | 54 |
| Triton X-100 + high salt | 98 | 2 |

A plasma membrane fraction (Fig. 4) using this procedure, we were able to obtain a highly purified plasma membrane fraction (Fig. 4). This method is based on the coating of 35S-labeled yeast spheroplasts with the lectin ConA that binds glycoproteins and selectively labels plasma membranes of intact cells. After cell lysis, the dense ConA-coated plasma membrane sheets can be separated from other cellular membranes by low speed centrifugation yielding the plasma membrane (PM) fraction. The low speed supernatant fraction is further separated into the particulate P100 and the cytosolic S100 fractions. By using this procedure, we were able to obtain a highly purified plasma membrane fraction (Fig. 4A) that contained only minor contamination by other organelle markers that was likely due to the presence of a small amount of unbroken cells in this fraction as evidenced by the cytosolic marker hexokinase. The majority of Tor1p and Tor2p copurified with plasma membrane. 50–65% of Tor1p and Tor2p was recovered in the PM fraction, whereas approximately 35–40% of Tor1p and Tor2p partitioned into the P100 fraction (Fig. 4A). Only trace amounts (5–10%) of Tor1p and Tor2p were detected in the cytosolic S100 fraction. We therefore conclude that more than half of Tor1p and Tor2p associates with the plasma membrane. The remaining portion of Tor is found in a different, high buoyant density fraction.

Tor1p and Tor2p Localization Is Rapid and Does Not Require Vesicle Transport—The presence of Tor1p and Tor2p in two distinct fractions may be explained by Tor passaging from one compartment to another. Alternatively, Tor1p and Tor2p could simultaneously associate with two compartments upon synthesis. To differentiate between these two possibilities, pulse-chase experiments were carried out with a labeling period of 1 min and increasing periods of chase with unlabeled amino acids. After the chase, cells were converted to spheroplasts and coated with ConA in the presence of cycloheximide to prevent new protein synthesis. Extracts were then generated and fractionated into PM, P100, and S100 fractions. We found that newly synthesized Tor1p and Tor2p rapidly partitioned into the PM and the P100 fractions (Fig. 4B). The majority of Tor1p and Tor2p was found in the PM and the P100 fractions even after only a 1-min pulse. The integral plasma membrane H+ -ATPase Pma1p, in contrast, which is transported to the cell surface via the secretory pathway, starts to accumulate significantly in the PM fraction only after 15–30 min of chase (Fig. 4B).

We also analyzed the subcellular distribution of Tor1p and Tor2p in a set of mutants defective in various stages of the secretory (sec4), endocytic (end4), or Golgi to vacuole (vps21, vps27, vps34, and vps45) protein transport pathways (36–38). Cells were shifted to the nonpermissive temperature for 1 h to induce the mutant phenotype, pulse-labeled, lysed, and fractionated by differential centrifugation and velocity gradient centrifugation as described above. The intracellular distribution of Tor1p and Tor2p was analyzed and compared with that in wild-type cells. We found no changes in the fractionation profiles of Tor1p or Tor2p in these mutants (data not shown). Thus, together with our findings that functional vesicular transport pathways are not required for normal Tor localization, we conclude that Tor1p and Tor2p associate with both subcellular sites most likely via a soluble intermediate.

Rapamycin Does Not Affect the Subcellular Localization of Tor1p and Tor2p—We next investigated whether treatment of yeast cells with rapamycin alters the intracellular distribution of Tor1p and Tor2p. Wild-type cells were labeled with 35S-Met/Cys for 20 min and chased with excess unlabeled amino acids in the presence of rapamycin (200 ng/ml) or carrier (ethanol) for 1 or 2 h at 30 °C. Microscopic examination of rapamycin-treated cells confirmed the effectiveness of rapamycin. After 1 h of rapamycin treatment, cells exhibited a marked increase in size and contained a single enlarged vacuole. After 2 h of treatment, cells were primarily arrested as large unbudded cells with large vacuoles (data not shown) as reported previously (4). Rapamycin-treated cells were converted to spheroplasts and coated with ConA to label the plasma membrane. ConA-coated plasma membrane (PM), particulate P100, and cytosolic S100 fractions were isolated and probed for the presence of Tor1p and Tor2p. Alternatively, extracts derived from rapamycin-treated cells were subjected to differential centrifugation and velocity gradient fractionation, and the fractionation profile of Tor1p and Tor2p was compared with that of cells treated with carrier alone. We found, by both methods, that rapamycin treatment did not affect Tor localization (Fig. 4B and data not...
shown). Interestingly, rapamycin did appear to delay the transport of Pma1p to the cell surface indicating that the drug might affect the rate of secretion (Fig. 4B).

Localization of Epitope-tagged Tor1p and Tor2p by Immunofluorescence—We also used immunofluorescence as an independent method to localize Tors. Because our initial attempts to detect endogenous Tor proteins or GFP-Tor expressed under control of the endogenous promoter failed, Tor1p and Tor2p were tagged with the HA epitope at their N termini. The HA-Tor2p fusion protein complements the lethality of a tor2 knock-out mutant demonstrating that the tag does not interfere with Tor2p function. HA-Tor1p was previously shown to be functional in vivo (39). Furthermore, HA-Tor2p cofractionated with endogenous Tor2p during differential centrifugation and in sucrose density gradients, demonstrating that overexpression does not significantly alter subcellular localization (data not shown). Thus, HA-Tor should accurately reflect the localization of the endogenous protein.

Visualization of HA-Tor1p and HA-Tor2p using monoclonal anti-HA antibodies revealed a discrete punctate staining at the cell periphery, as well as a less pronounced internal dot-like staining (Fig. 5A, and data not shown). No signal was detected in yeast cells overexpressing untagged Tor protein (data not shown). Comparison of the anti-HA and DAPI stainings showed that HA-Tor1p and HA-Tor2p do not localize to the nucleus or the perinuclear area (Fig. 5B, and data not shown). Furthermore, neither HA-Tor1p nor HA-Tor2p appears to colocalize with the vacuolar marker alkaline phosphatase (Fig. 5, D and E). Confocal microscopy demonstrated that the majority of HA-Tor1p and HA-Tor2p is localized to the plasma membrane, whereas the remaining portion is distributed throughout the cell interior in a dot-like pattern (Fig. 5, F and G). Thus, our immunofluorescence studies are in agreement with our biochemical fractionation studies and suggest that Tors are associated with the plasma membrane and another, as yet

*J. Kunz, U. Schneider, I. Howald, A. Schmidt, and M. N. Hall, unpublished results.
unidentified, location.

Two Regions Containing HEAT Repeats Independently Mediate Tor2p Localization—To define the region(s) within Tor2p that mediates localization, we constructed deletion variants of Tor2p, and we tested their ability to associate with the particulate P13 and P100 fractions (summarized in Fig. 6). We found that an N-terminal fragment containing amino acids 1–326 (Tor2p\textsuperscript{D327–2474}) was sufficient for Tor2p localization. This fragment appeared to be unstable in yeast, yet associated with the P13 and P100 fractions in a manner similar to the wild-type protein. In accordance with this, an internal deletion mutant, which contained the N-terminal 326 amino acids but lacked a central domain (Tor2p\textsuperscript{D327–1389}), was stable and behaved identically in fractionation experiments. The C-terminal kinase homology domain, as well as the FKBP12-rapamycin binding domain, however, were not required for association of Tor2p with the P13 and P100 fractions (Tor2p\textsuperscript{D1690–2474} and Tor2p\textsuperscript{D1390–2474}).

The N-terminal domain is sufficient but not necessary to mediate localization. Deletion of amino acids 1–326 did not result in mislocalization of Tor2p (Tor2p\textsuperscript{D327–1386}). This indicates that a second domain within Tor2p independently mediates membrane association and localization. This second region is located between amino acids 327 and 1690, since a deletion mutant (Tor2p\textsuperscript{D1–1690}) lacking this region failed to fractionate with the P100 fraction and partitioned entirely into the P13 fraction. This could have indicated that this truncation mutant still associates with the plasma membrane but lacks the information for localization to the second compartment. However, when analyzed by equilibrium density gradient centrifugation, Tor2p\textsuperscript{D1–1690} failed to cofractionate with wild-type Tor2p but rather segregated into a denser part of the gradient (data not shown). This suggests that the information for proper subcellular localization is lost in this mutant and that the protein aberrantly associates with another subcellular compartment. Further analysis of Tor2p deletion variants (Tor2p\textsuperscript{D1390–2474}, Tor2p\textsuperscript{D1690–2239}, and Tor2p\textsuperscript{D1390–1945}) restricted the second domain contributing to Tor2p localization to a region between amino acids 487 and 1690. In summary, these data indicate that localization of Tor2p involves two regions that do not include the kinase domain or the FKBP12-rapamycin-binding site but contain extensive HEAT repeats, termed HEAT repeat domains that can independently mediate association of Tor2p with both intracellular sites.

DISCUSSION

We investigated the subcellular distribution of Tor1p and Tor2p by biochemical and immunofluorescence approaches, and we found that Tor1p and Tor2p similarly distribute to two biochemically distinct fractions. We have identified one of these fractions as the plasma membrane. The majority of Tor1p and Tor2p cofractionated with the integral plasma membrane...
tor localization

fig. 5. intracellular localization of tor1p and tor2p. wild-type yeast cells transformed with vectors containing ha-tor1 or ha-tor2 under the control of the inducible gali promoter were grown at 30 °c in medium containing glycerol as the carbon source and then shifted to medium containing galactose to induce expression of the fusion protein. cells were incubated for 4–5 h at 30 °c before being fixed and processed for immunofluorescence. a, indirect immunofluorescence showing ha-tor2p; b, ha-tor2p (green) overlaid with dapi staining (purple) to visualize nuclei; c, nomarski optics to visualize cells; d, ha-tor1p (green) overlaid with alkaline phosphatase staining (red) to visualize vacuoles; e, ha-tor2p (green) overlaid with alkaline phosphatase staining (red); f, confocal slice of cells expressing ha-tor1p; g, confocal slice of cells expressing ha-tor2p.

marker pma1p during differential centrifugation studies and on sucrose density equilibrium gradients. association with plasma membrane was further substantiated by an independent procedure using the lectin cona that selectively binds to glycoproteins on intact yeast spheroplasts and allows the purification of highly enriched plasma membrane sheets. 50–65% of tor1p and tor2p was found to purify with plasma membrane, whereas other organelle markers could be clearly separated from this fraction. in agreement with the biochemical data, immunofluorescence staining of ha-tor1p and ha-tor2p was detected at discrete sites at the cell periphery and in a punctate pattern throughout the cytoplasm.

the localization of tor1p and tor2p to the plasma membrane is consistent with their function. one function of tor2p is to control organization of the actin cytoskeleton by activation of a gtpase switch composed of rho1p, rho2p, and the gdp/gtp exchange factor rom2p (12, 13). a second function of tor2p that is shared with tor1p is to regulate translation initiation and g1 progression in response to nutrient availability in yeast. both rom2p and rho1p have been localized to the plasma membrane, and rho1p in addition has also been detected on golgi structures and post-golgi vesicles (40–42). furthermore, mss4p, an essential phosphatidylinositol 4-phosphate 5-kinase and candidate component of the tor signaling pathway, is also localized to the plasma membrane (21, 43, 44). in addition, plc1p, the yeast homolog of phospholipase c, has been linked to both tor2p functions and may mediate generation of lipid second messengers at the plasma membrane (21, 30). the localization of tor proteins to the plasma membrane is therefore consistent with the localization of known or suspected downstream effectors.

how localization of tor1p and tor2p to a second subcellular site may relate to their function as well as the nature of this site remains to be determined. our subcellular fractionation and indirect immunofluorescence studies demonstrate that this second site is distinct from mitochondria, nuclei, golgi, and vacuoles. further studies will be required to determine whether tor proteins associate with membranes of high buoyant density or with a large protein complex, both of which could be expected to partition into the p100 fraction.

cardenas et al. (45) have reported previously that tor2p is localized to the surface of yeast vacuoles and that this association is disrupted by the immunosuppressive drug rapamycin. this is in contrast to our results, which show that tor proteins are localized to the plasma membrane and a second compartment, which is biochemically distinct from the vacuole. furthermore, we do not observe an effect of rapamycin on tor localization. the reason for the discrepancy between their results and ours is not known at present. however, we base our interpretation on several observations. (a) we have demonstrated that our antisera specifically recognize endogenous tor1p or tor2p and do not cross-react. (b) our biochemical fractionation data are consistent with findings obtained by direct purification of plasma membrane and results obtained by immunofluorescence studies using epitope-tagged tor1p and tor2p. (c) we do not observe any immediate effects of rapamycin on tor2p localization as judged by several independent methods including differential centrifugation, sucrose density gradient centrifugation, purification of plasma membrane, and indirect immunofluorescence.

how do tor proteins become membrane-bound and reach their subcellular location? our extraction data suggest that at least part of tor1p and tor2p are peripheral membrane proteins. only reagents that disrupt protein-protein interactions efficiently extract the tor proteins into a soluble fraction. the reproducible effect of detergent extraction suggests that the association of tor1p and tor2p with membranes may be complex and may involve both ionic and hydrophobic protein-protein interactions with a membrane-bound protein(s) or even protein-lipid interactions. pulse-chase experiments indicate that tor1p and tor2p associate rapidly and simultaneously with their final destinations and that this association is most likely via a soluble intermediate. this is in accordance with our finding that tor localization is not altered in yeast mutants that are defective for endocytic, secretory, or golgi to vacuole vesicle trafficking pathways. thus, association of tor proteins with membranes is likely mediated by interaction with an integral membrane protein or with a membrane-associated protein complex.

we used deletion analysis of tor2 to identify the region(s) that mediate localization and, potentially, may engage in protein-protein interaction. this analysis identified two domains, in the n terminus and center of tor2p, that independently mediate localization. the most prominent feature in these domains is an extensive stretch of sequence composed of a repeated antiparallel a-helical motif, termed heat repeat motif (46). these repeats extend in two blocks (amino acids 100–470 and 560–1220) and encompass the two regions identified by our deletion analysis as sufficient for mediating localization. because tor1p colocalizes with tor2p and also contains heat repeats (1, 46), it is likely that similar findings apply to its localization.

heat repeat motifs are highly divergent and have been detected in a number of proteins, including huntingtin, elongation factor 3, the 65-kda structural a subunit of protein phosphatase 2a (pp2a r65/a), the nuclear pore transport protein importin b, and the splicing factor sap155, for example, many of which have a demonstrated or suspected role in vesicular or protein traffic and form multiprotein complexes (46, 47). the three-dimensional structure of the heat repeats in several of these proteins has been recently solved and reveals an extended curved conformation composed of a double layer of...
α-helices (48–51). This structure provides a large exposed surface with a hydrophobic nature that can participate in multiple protein-protein interactions. Structural data in combination with biochemical and deletion analysis indicate that groups of HEAT repeats indeed mediate interactions with distinct binding partners (48–51).

HEAT repeats domains may, therefore, anchor Tor to the membrane by mediating interactions with membrane-associated protein(s). The large and extended surface formed by these repeats may also provide additional protein-protein interaction interfaces with effectors or regulatory components. In accordance with such a hypothesis, we found that overexpression of HEAT repeats indeed mediate interactions with distinct binding partners (48–51).

Striking parallels also exist between Tor2p and other proteins containing HEAT repeats. Huntingtonin, the protein affected in Huntington’s disease, is a large protein that is localized to multiple subcellular compartments, including vesicular structures, and interacts with a plethora of proteins involved in actin cytoskeleton function and possibly vesicular transport (47). Furthermore, ATM, a Tor-related protein, is localized to the nucleus and cytoplasmic vesicles and was recently shown to bind β-adaptin, a protein involved in clathrin-mediated endocytosis (52).

It is therefore tempting to speculate that HEAT repeats in Tor proteins act as molecular scaffolds for the recruitment of signaling complexes. The large interaction surface in these proteins and their localization to multiple cellular compartments may provide a means to integrate several inputs and to relay signals regulating temporal as well as spatial growth. An important issue now becomes identification of Tor-interacting protein(s). Definition of minimal domains that mediate localization should facilitate the isolation of protein(s) that anchor Tor to membranes and may potentially lead to the identification of regulators and additional effectors of the Tor pathway.

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FIG. 6. Identification of Tor2p domains that mediate association with the particulate fractions. A schematic representation showing Tor2p and the boundaries of Tor2p deletion mutants. The structure of Tor2p is presented on top. Regions indicated are the HEAT repeat-containing domains (HEAT), the FKBP12-rapamycin-binding domain (RBD), the kinase homology domain (KD), and the cysteine-rich motif at the C terminus (CYS). The region present in each deletion mutant is depicted below. Amino acid residues present or lacking in these mutants are indicated with + and − signs. PhosphorImager. Plus and minus signs indicate the ability or inability, respectively, of each mutant to associate with P13 and P100 fractions in a manner quantitatively similar to the wild-type protein.

Tor Localization
HEAT Repeats Mediate Plasma Membrane Localization of Tor2p in Yeast
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