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Tor2 Directly Phosphorylates the AGC Kinase Ypk2 To Regulate Actin Polarization†

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The target of rapamycin (TOR) protein kinases, Tor1 and Tor2, form two distinct complexes (TOR complex 1 and 2) in the yeast *Saccharomyces cerevisiae*. TOR complex 2 (TORC2) contains Tor2 but not Tor1 and controls polarity of the actin cytoskeleton via the Rho1/Pkc1/MAPK cell integrity cascade. Substrates of TORC2 and how TORC2 regulates the cell integrity pathway are not well understood. Screening for multicopy suppressors of *tor2*, we obtained a plasmid expressing an N-terminally truncated Ypk2 protein kinase. This truncation appears to partially disrupt an autoinhibitory domain in Ypk2, and a point mutation in this region (Ypk2^{D239A}) conferred upon full-length Ypk2 the ability to rescue growth of cells compromised in TORC2, but not TORC1, function. *YPK2*^{D239A} also suppressed the lethality of *tor2*Δ cells, suggesting that Ypks play an essential role in TORC2 signaling. Ypk2 is phosphorylated directly by Tor2 in vitro, and Ypk2 activity is largely reduced in *tor2*Δ cells. In contrast, Ypk2^{D239A} has increased and *TOR2*-independent activity in vivo. Thus, we propose that Ypk protein kinases are direct and essential targets of TORC2, coupling TORC2 to the cell integrity cascade.

Cell growth and proliferation are tightly linked with the cell's perception of its nutritional environment. Tor (target of rapamycin) proteins belong to a family of phosphatidylinositol kinase-like protein kinases and play a central role in controlling cell growth (12, 17). Specifically, TOR signaling couples nutrient signals to various growth-related processes, including protein synthesis, gene expression, uptake of amino acids, actin organization, endocytosis, and autophagy.

The budding yeast *Saccharomyces cerevisiae* has two homologous *TOR* genes, *TOR1* and *TOR2*, the protein products of which regulate at least two essential signaling branches (17). In one branch, Tor1 and Tor2 act redundantly to control temporal aspects of cell growth; i.e., when adequate nutrients are available Tor promotes the accumulation of mass by increasing translation (2, 5, 25) and ribosome biogenesis (30) and by repressing autophagy (20). TOR signaling in this branch is sensitive to rapamycin. Tor2 but not Tor1 also functions in a second essential signaling branch. In this branch Tor2 controls spatial aspects of cell growth by regulating the cell cycle-dependent polarization of the actin cytoskeleton. Polarization of the actin cytoskeleton is thought to orient the secretory apparatus such that newly synthesized macromolecules are efficiently delivered to the major site of growth–the bud. A distinct TOR complex operates in each of these signaling branches (26). TOR complex 1 (TORC1) contains Kog1, Tco89, Lst8, and either Tor1 or Tor2. TORC1 regulates the temporal aspects of cell growth, and Tor in TORC1 is bound and inhibited by rapamycin. TORC2 contains Avo1, Avo2, Avo3, Bit61, Lst8, and Tor2 but not Tor1. TORC2 regulates the spatial aspects of cell growth, and Tor2 in TORC2 is not bound by rapamycin; therefore, TORC2 function is insensitive to this drug.

In wild-type yeast, actin patches are concentrated in small- and medium-sized buds and are simultaneously absent from the mother cell. Disruption of *TOR2* or TORC2 results in a loss of this actin patch polarization (34, 35). Recently, receptor internalization was also observed to be impaired in *tor2* mutants (7). TORC2 acts upstream of the Rho1 GTPase, presumably by regulating the activity of the Rho1 GTP/GDP exchange factor Rom2 (26, 34). Rho1 has several known effectors. One Rho1 effector is the yeast protein kinase C homologue Pkc1. Pkc1 acts upstream of the "cell integrity" mitogen-activated protein kinase (MAPK) pathway (21), and this pathway is required for TORC2/Rho1 to regulate actin polarization (13). A second Rho1 effector is the glucan synthase Fks1. Fks1 appears to act downstream of TORC2/Rho1 to regulate receptor internalization (7). However, the mechanisms by which TORC2 regulates Rom2 are unknown.

Substrates of mammalian TOR (mTOR) have been reported, the best characterized being the translation regulators 4E-BP1 and S6K1 (12). S6K1 belongs to a family of related kinases known as AGC kinases. Recently, Matsuo et al. reported that overexpression of the AGC kinase *gad8* rescues the

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† This paper is dedicated to the memory of Shoshi Muto, whose mentorship meant a great deal to Y.K.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
YMW1	*MAT*α *ade2-1 ade3-Δ22 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	Laboratory stock
YYK241	YMW1 *tor2* p414GAL1[*Tor2 ADE3*]	This study
W303	*MAT***a**/α *ade2 his3 leu2 trp1 ura3 can1*	Y. Mukai
W303-1A	*MAT*α *ade2 his3 leu2 trp1 ura3 can1*	Y. Mukai
W303-1B	*MAT***a** *ade2 his3 leu2 trp1 ura3 can1*	Y. Mukai
DL201	*MAT***a** *his4 leu2 ura3 ypk1*Δ::*TRP1*	D.E. Levin
YYK346	W303-1B *ypk1*Δ::*TRP1*	This study
YYK373	W303-1B *ypk1*Δ::*TRP1 ypk2*Δ::*KanMX* p415GAL1[*Ypk2-244*]	This study
YYK339	W303 *tor1*Δ::/*KanMX*/*Tor1 tor2*Δ::*HIS3*/*Tor2*	This study
YYK354	W303-1B *tor2*Δ::*HIS3* pRS316[*Tor2*]	This study
YYK357	W303-1B *tor1*Δ::*KanMX tor2*Δ::*HIS3* pRS316[*Tor2*]	This study
JK9-3da	*MAT***a** *his4 leu2-3, 112 ura3-52 trp1 rme1 GAL*$^+$	13
SH100	JK9-3da *tor2*Δ::*ADE2-3* YCplac111[*Tor2*]	13
SH121	JK9-3da *tor2*Δ::*ADE2-3* YCplac111[*tor2-21*^{ts}]	13
SH221	JK9-3da *tor1*Δ::*HIS3-3 tor2* Δ::*ADE2-3* YCplac111[*tor2-21*^{ts}]	13
INA28-1B	*MAT***a** *ade1 his2 leu2 trp1 ura3 pkh2*Δ::*LEU2*	15
INA106-3B	INA21-1B *pkh1-D398G*^{ts}	15
TB50a	*MAT***a** *his3 leu2-3,112 trp1 ura3 rme1*	Laboratory stock
RL23-1c	TB50a [KanMX4]-GAL1p-*AVO1*	26
RL93a	TB50a [KanMX4]-GAL1p-*KOG1*	26

mating deficiency elicited by a *tor1* mutation in fission yeast (27). It is important to note that fission yeast strains harboring mutations in *TOR1*, or in genes encoding proteins homologous to budding yeast TORC2 components, all share very similar phenotypes. For example, *ste20* (= *Sc AVO3*), *sin1* (= *Sc AVO1*), and *tor1* strains all have very similar mating defects and all are hypersensitive to various stresses (14, 22, 37, 38). These observations suggest that fission yeast tor1 more closely resembles budding yeast Tor2 and may therefore function primarily in a TORC2-like complex.

Budding yeast encodes a pair of GAD8 orthologues, *YPK1* and *YPK2*, that perform a redundant, essential function (4). The protein kinase activities of the Ypks are dependent upon the *PKH1* and *PKH2* genes (31), and Ypk1 is directly phosphorylated and activated by Pkh1 (3). *PKH* genes encode PDK1-like protein kinases (15). While mammal PDK1 requires 3-phosphoinositide as an activating cofactor, Pkhs are activated by the presence of sphingolipids (8). Overexpression of YPK1 confers resistance to ISP-1, an analog of sphingosine that inhibits the serine-palmityl transferase LCB1 and thus blocks sphingolipid production, confirming that Ypks function downstream of Pkhs (36). Recently, several reports have demonstrated that Ypks and TORC2 share similar downstream readouts. For example, receptor internalization and fluid-phase endocytosis are impaired in *ypk* mutants (6) and loss of Ypk function results in loss of actin polarization (31, 33). Furthermore, overexpression of Rho1 exchange factor (*TUS1*) suppresses the lethality of both *ypk* mutants and TORC2 mutants, suggesting that Ypks act in parallel or downstream of TORC2 signaling (33).

In a screen for multicopy suppressors of the lethality of a *tor2* mutant, we obtained a 5′-truncated allele of *YPK2*. A point mutation near the truncated region (e.g., D239A mutation) conferred upon full-length *YPK2* the ability to act as a *tor2* suppressor. We further demonstrated that immunopurified Tor2 directly phosphorylates Ypk2 in vitro. In vivo, phosphorylation of Ypk2 by Tor2 is required for both Ypk2 kinase activity and function. In contrast, Ypk2^{D239A} has increased and *TOR2*-independent activity, and *YPK2*^{D239A} can suppress the lethality resulting from loss of TORC2 function but not loss of TORC1 function. Together, these results indicate that Ypk2 is a substrate of TORC2 and is required for TORC2 to regulate spatial aspects of cell growth.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. Standard techniques were used for yeast manipulation (19). Yeast strains used in this study are listed in Table 1. YYK241 was created by chemical mutagenesis of YMW1. Mutation in the *TOR2* locus was confirmed by integration mapping (data not shown). Deletion constructs to disrupt *YPK2* and *TOR1* with KanMX were amplified by PCR from BY4741-based deletion mutants (10). YYK346 was constructed by crossing DL201 and W303-1A three times into the W303 genetic background. Deletion *TOR2* with *HIS3* was constructed as follows. A 2.6-kb fragment containing 2.4 kb of the 3′ region of *TOR2* open reading frame was amplified by PCR and cloned into BamHI and XhoI sites of pBluescriptII KS+. The *HIS3* gene (1.3 kb) was then used to replace a 0.9-kb SalI-BglII fragment, yielding a *tor2*Δ::*HIS3* cassette. The resultant deletion cassette was transformed into a W303 diploid strain. Similarly, a *TOR1* deletion cassette (*tor1*Δ::KanMX) was transformed into W303 to make YYK339. YYK339 was transformed with pRS316[*TOR2*], and its segregants generated YYK354 and YYK357. Site-directed mutagenesis was done using a QuikChange site-directed mutagenesis kit (Stratagene).

Multicopy suppressor screening. The YYK241 strain was transformed with a yeast genomic DNA library constructed in the YEp24 multicopy plasmid, and the transformants were grown on selective medium (SC)-galactose-Ura plates. We screened approximately 24,000 colonies, and we picked 27 white sectoring colonies as the first candidates. We further examined their growth at 37°C (nonpermissive temperature of YYK241 cells) on YEPD as a second screening. We were left with six candidates as multicopy suppressors of YYK241 cell. Genomic library-derived plasmids were rescued, and the genes contained in these plasmids were identified by sequencing both ends of the inserts. Deletion and subcloning analyses revealed that the 5′-truncated *YPK2* gene was a suppressor.

Protein kinase assay of Tor2 protein. SH121cell harboring pRS314[^{HA}*TOR2*] plasmid (18) grown in yeast extract-peptone-dextrose (YEPD) medium were collected, washed once with distilled water, and suspended in ice-cold TOR lysis buffer (16 μl/optical density at 600 nm cell) (1× phosphate-buffered saline [PBS] [pH 7.4], 10% glycerol, 4 mM Na_3VO_4, 50 mM KF, 15 mM Na-PPi [pH 7.5], 15 mM *p*-nitrophenylphosphate [*p*NPP], 20 μg/ml leupeptin, 20 μg/ml benzamidine, 10 μg/ml pepstatin A, 40 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF]). An equal volume of glass beads (425 to 600 μm) was added to this suspension, and cells were broken by vigorous vortexing for 10 min at 4°C. A half volume of lysis buffer containing 2% Tween 20 was added, and the mixture was further incubated for 10 min with mild rotation. The beads and cell debris were

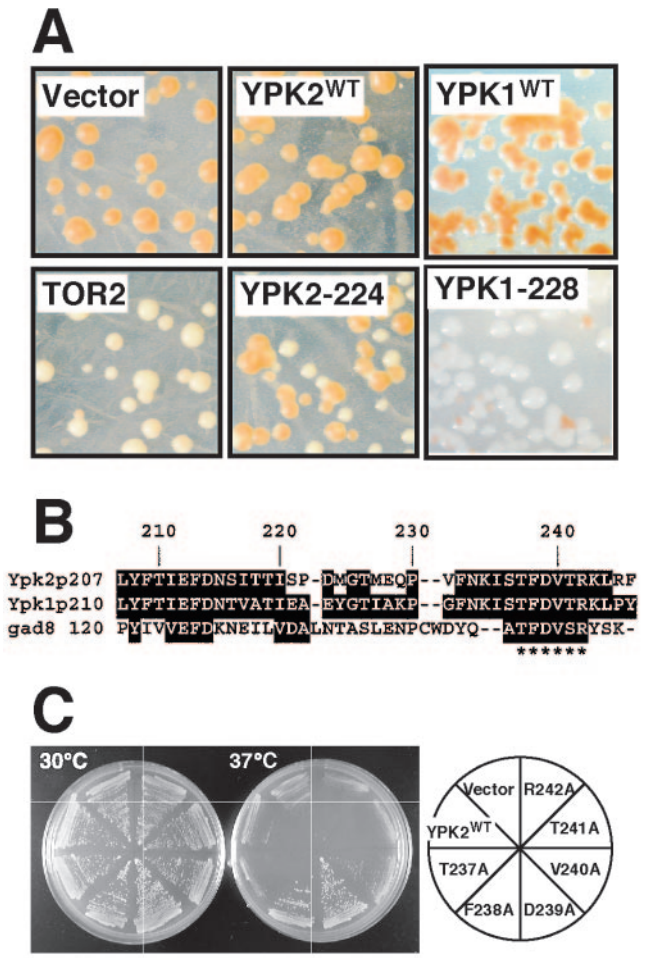

FIG. 1. N-truncated Ypk2 suppresses *tor2* mutants. A. YYK241 (*ade2 ade3 tor2* p414GAL1[*TOR2 ADE3*]) was transformed with YEp352 (vector), YEp352[full-length *YPK2*] ($YPK2^{FL}$), YEp352[N-truncated *YPK2*] (YPK2-224), and YEp352[N-truncated *ypk2*-K373A (kinase dead)] ($YPK2\text{-}224^{KD}$), as indicated. The transformants were grown on YEPD plates for 4 days at 30°C. Sectoring colonies showed independence of *TOR2* plasmid, i.e., suppression of *tor2* mutation. B. Alignment of the amino acid sequences around the M224 of Ypk2. Ypk1, Ypk2 (*S. cerevisiae*), and Gad8 (*S. pombe*) are shown. The arrowhead highlights M224 of Ypk2, and asterisks denote the conserved region shared among these protein kinases. C. *YPK2* genes (cloned into YEp352) harboring a point mutation in the conserved region as indicated (right panel) were transformed into YYK241, and the resultant transformants were grown on YEPD plates for 2 days at 30°C (left) or 37°C (right). Note that these *YPK2* genes expressed full-length of Ypk2 protein under the control of their own promoters.

removed by centrifugation for 10 min at 10,000 × *g* at 4°C, and the supernatant was further clarified by an additional 10-min centrifugation. Glycerol was added to the resulting lysate to a final concentration of 30% before storage at −20°C. Protein concentration was estimated using a BCA kit (Pierce). For immunoprecipitation of ^{HA}Tor2 protein, 1 mg of cell lysate was brought up to 1 ml with immunoprecipitation buffer (TOR lysis buffer containing 0.5% Tween 20 and 0.25% gelatin) and incubated with 1 μl of antihemagglutinin (anti-HA) ascites (16B12; BabCO) for 2 h at 4°C with gentle rotation. To the extract/antibody mixture was added 20 μl of 50% suspension of protein G-Sepharose 4FF (Amersham), followed by a further rotation for 1 h at 4°C. Immunocomplexes were transferred into fresh microcentrifuge tubes and washed four times with 1 ml of immunoprecipitation buffer (without gelatin) with gentle rotation and twice with 1 ml of 25 mM HEPES-KOH (pH 7.5). The resultant immunocomplexes were resuspended in 36 μl of TOR kinase assay buffer (25 mM HEPES-KOH [pH 7.5], 50 mM NaCl, 10 mM $MnCl_2$, 15 mM *p*NPP) containing 4 μg of substrates (4E-BP1 [Santa Cruz] or recombinant Ypk2). This mixture was preincubated for 3 min at 30°C before the reaction was initiated by adding 4 μl of 0.5 mM [γ-^{32}P]ATP (Amersham) (20 μCi/sample). After incubation for 60 min at 30°C, the reaction was terminated by addition of 40 μl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and incubation for 5 min at 65°C. Samples were subjected to 7.5 to 12.5% SDS-PAGE gels. After electrophoresis, gels were immersed in 12.5% trichloroacetic acid for 30 min, followed by several washes in distilled water. Proteins were detected by Coomassie staining (GelCode Blue; Pierce). Phosphorylated proteins were detected by autoradiography.

Protein kinase assay of Ypk2 protein. C-terminal hemagglutinin (HA) tagging of Ypk2 ($Ypk2^{HA}$) was conducted as follows. The EcoRI-EcoRI fragment (2.4 kb) of YEp352[*YPK2*] plasmid (4) was cloned into pUC18. A NotI site was created immediately 5′ to the translational terminator by use of a QuikChange kit (Stratagene). Next, a NotI cassette of 3× HA was subcloned into the newly created NotI site of *YPK2*. For immunoprecipitation experiments, cells harboring YEp352[$YPK2^{HA}$] plasmid grown in YEPD were collected, washed once with distilled water, and suspended in ice-cold YPK lysis buffer (1× PBS [pH 7.4], 1 mM EDTA, 1 mM EGTA, 4 mM Na_3VO_4, 50 mM KF, 15 mM Na-PPi [pH 7.5], 15 mM *p*NPP, 20 μg/ml leupeptin, 20 μg/ml benzamidine, 10 μg/ml pepstatin A, 40 μg/ml aprotinin, 1 mM PMSF). Cells were broken by vortexing with glass beads as described above. The beads and cell debris were removed by centrifugation for 10 min at 10,000 × *g* at 4°C, and the supernatant was further clarified by an additional 10-min centrifugation. Glycerol was added to the resulting lysate to a final concentration of 30% before storage at −20°C. $Ypk2^{HA}$ protein was immunoprecipitated as described above with several modifications. The immunoprecipitation buffer used was YPK lysis buffer containing 0.5% Tween 20 (and it did not contain gelatin), immunoprecipitation was conducted with 100 to 1,000 μg protein in a 500-μl volume, and incubation time with HA antibody was 5 h. Immunoprecipitated $Ypk2^{HA}$ protein was suspended with 18 μl of YPK kinase assay buffer (50 mM MOPS-KOH [pH 7.5], 10 mM $MgCl_2$, 1 mM Na_3VO_4) containing 4 μg 2× crosstide (GRPRTSSFAEGGRPRTSSFAEG). This mixture was preincubated for 3 min at 30°C, and the reaction was initiated by adding 2 μl of 1 mM [γ-^{32}P]ATP (Amersham) (10 μCi/sample). After incubation for 30 min at 30°C, the reaction was terminated by addition of 30 μl of 2× SDS-PAGE sample buffer and incubation for 5 min at 65°C. Samples were subjected to 15% SDS-PAGE gel. After electrophoresis, gels were immersed in 12.5% trichloroacetic acid for 30 min, followed by several washes in distilled water. Proteins were detected by Coomassie staining (GelCode Blue; Pierce). Phosphorylated proteins were detected by autoradiography or BAS2000 (Fuji Film), the amount of immunoprecipitated $Ypk2^{HA}$ was monitored by immunoblotting (LAS1000; Fuji Film), and the specific activity of Ypk2 was estimated from these values.

Actin staining. YEPD-grown cells were shifted from 30°C to 37°C for 2 h. Cells were then fixed for 30 min by the direct addition of 37% formaldehyde stock to a final concentration of 5%. Fixed cells were collected, washed with 1× PBS three times, and then 0.1 U/μl of rhodamine-phalloidin (Sigma) was added for 2 h at room temperature to stain actin as described previously (19). Cells were observed, and the images were acquired as described previously (28) using a rhodamine filter.

Preparation of recombinant Ypk2 protein. We used modified pGEX-6p-1 (Amersham) for expression of recombinant protein. An annealed oligonucleotide (5′-TCGACTCCACCATCATCATCATTAATGC-3′) was inserted into the SalI-NotI sites of pGEX-6p-1, and we designated the created plasmid pGEX-His. A YPK2 open reading frame was amplified with primers 5′-GGGG ATCCATGCATTCCTGGCGAATATC-3′ and 5′-ACGCGTCGACCACTAAT GCTTCTCCCCTGC-3′, with YEp352[*YPK2*] as a template. The PCR product was cloned into BamHI-SalI sites of pGEX-His (designated pGEX[YPK2-His]). To create pGEX[$YPK2^{S641A\ T659A}$-His]), a QuikChange kit (Stratagene) was used. The plasmids of YPK2 with an amino-terminal glutathione *S*-transferase-tagged and a carboxyl-terminal hexahistidine-tagged construct were transformed into *Escherichia coli* BL21(DE3). The cells were disrupted by sonication at 4°C in PBS (pH 7.4)–1 mM EDTA–5 mM dithiothreitol–0.1 mM PMSF. The protein was applied on a glutathione-Sepharose 4B column (Amersham) equilibrated with PBS (pH 7.4). The bound protein was eluted with 50 mM Tris-HCl (pH 8.0) and 5 mM reduced glutathione. The amino-terminal glutathione *S*-transferase tags in proteins were then removed by incubation with PreScission protease (Amersham) for 6 h at 4°C, and the digested proteins with a carboxyl-terminal His tag were applied on an Ni-nitrilotriacetic acid (NTA) column (QIAGEN) equilibrated with 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10 mM imidazole. The bound protein was eluted with 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 200 mM imidazole. Fractions containing proteins were purified on a

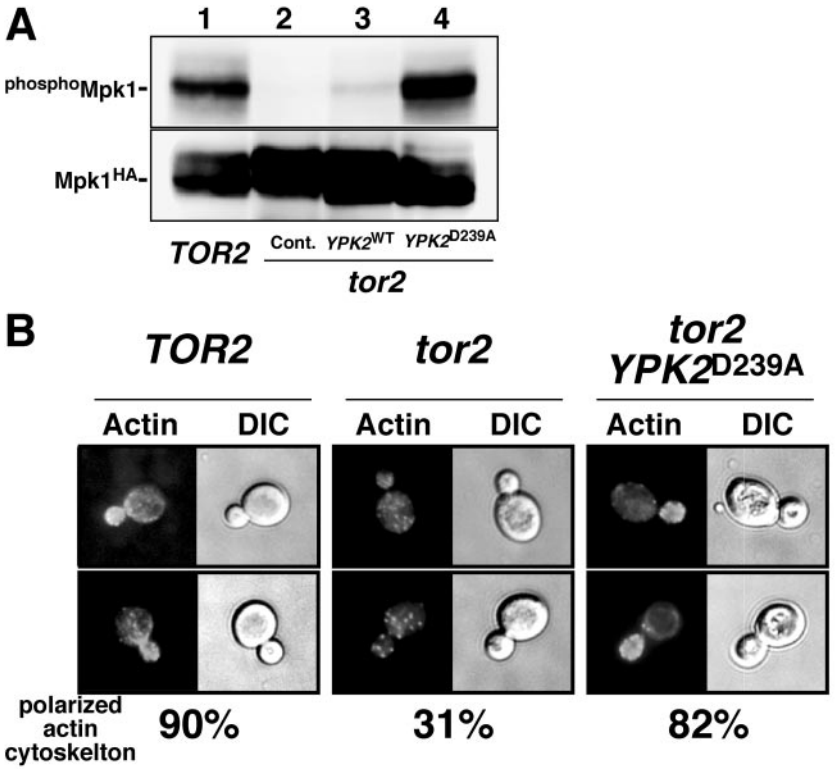

FIG. 2. *YPK2*^{D239A} restores Mpk1 activation and suppresses actin cytoskeleton organization defects in *tor2* mutant cells. A. Wild-type cells (YMW1) and *tor2* mutant cells (YYK241) harboring indicated YPK2 plasmids were transformed with pRS424[HA-tagged *MPK1* (MPK1^{HA})] to express Mpk1^{HA} protein. Cells were grown at 37°C, and phosphorylation of Mpk1^{HA} was determined by immunoblotting with anti-diphospho-p44/p42 MAPK (Cell Signaling Technology) (top panel). Total Mpk1 was determined by immunoblotting with anti-HA antibody (bottom panel). B. Wild-type (SH100) and *tor2-21* mutant (SH121) cells harboring YEp352[*YPK2*^{D239A}] grown in YEPD at 30°C were shifted to 37°C for 6 h. The actin cytoskeleton was stained with rhodamine-phalloidin as described in Materials and Methods. The percentage of cells exhibiting a polarized actin patch is also shown (bottom).

Superdex 200 gel filtration column (Amersham) and eluted with 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, and 150 mM NaCl.

RESULTS

To identify a novel factor(s) involved in TOR signaling, we screened for multicopy suppressors of the lethality of a *tor2* mutant. The yeast strain YYK241 (see Table 1) has mutations in the *ADE2*, *ADE3*, and *TOR2* loci and harbors plasmid p414GAL1[*TOR2 ADE3*]. This strain requires maintenance of the plasmid for growth and thus forms nonsectoring red colonies (Fig. 1A; *ade2* cells accumulate a red pigment, *ade2 ade3* cells do not). In galactose-containing medium *TOR2* is overexpressed, and YYK241 cells grow like wild-type cells. In glucose-containing medium YYK241 cells have a temperature-sensitive cell lysis phenotype (at 37°C; data not shown). We transformed YYK241 with a yeast genomic library, and from 24,000 transformants we obtained seven colonies that could form white sectors. By DNA sequencing and subcloning, we determined that a 5′ truncated *YPK2* gene suppressed the *tor2* mutant phenotype (Fig. 1A). The 5′-most methionine codon in the open reading frame of the truncated *YPK2* is M224, so we assume that the polypeptide produced starts at position 224. Hereafter we refer to this suppressor as *YPK2-224*. The remaining candidates contained the *TOR2* gene (data not shown).

We expected that overexpression of full-length *YPK2* would also suppress the *tor2* phenotypes. However, to our surprise, full-length *YPK2* did not act as a multicopy suppressor (*YPK2*^{FL}; Fig. 1A). Similarly, a kinase-dead (ATP-binding site) mutant, *YPK2*-224-K373A (*YPK2*-224^{KD}), was also unable to suppress *tor2* phenotypes, indicating that kinase activity of Ypk2-224 is required for the suppression. We further tried to examine kinase activity of Ypk2-224, but the expression level of the protein was too low for biochemical analyses (data not shown). Ypk2 functions redundantly with Ypk1. We therefore examined whether a 5′-truncated *YPK1* could also act as a *tor2* multicopy suppressor. Full-length and 5′-truncated *YPK*s were expressed using the GAL1 promoter in YYK241. Expression of 5′-truncated *YPK1* permitted sectoring, but overexpression of full-length *YPK1* did not (data not shown). As the truncated versions of *YPK1* and *YPK2* behave similarly, we focused on only the *YPK2* gene in subsequent experiments.

We hypothesized that a *cis*-inhibitory motif is compromised in the *YPK2-224* allele, which results in a polypeptide with increased kinase activity. We aligned the Ypk1, Ypk2, and Gad8 sequences to see whether there are any regions around Ypk2 aa224 that may be highly conserved. Indeed, there is a

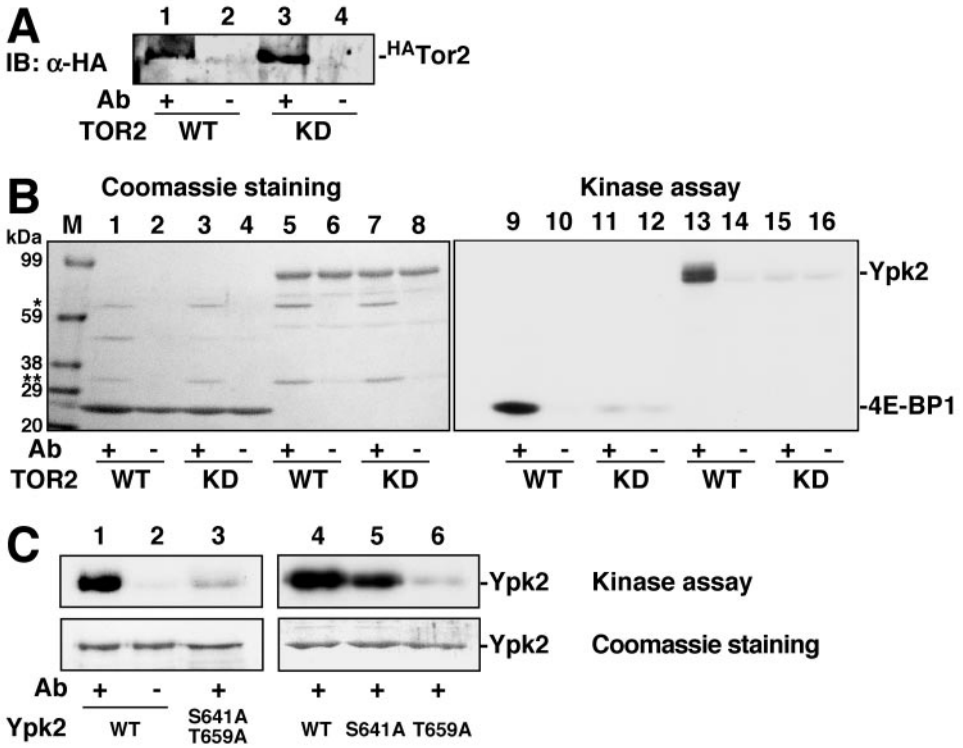

FIG. 3. Tor2 directly phosphorylates Ypk2 in vitro. A. Detection of immunoprecipitated HA-tagged Tor2 (^{HA}TOR2). ^{HA}Tor2$^{wild\ type}$ (WT; lanes 1 and 2) or ^{HA}Tor2^{D2298E} (kinase dead mutant [KD]; lanes 3 and 4) were immunoprecipitated in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of anti-HA antibody. Immunocomplexes were subjected to 7% SDS-PAGE gels, and ^{HA}Tor2 protein was detected by immunoblotting with anti-HA antibody. B. In vitro Tor2 kinase assay. Immunoprecipitated ^{HA}Tor2 (wild type [WT] or kinase dead [KD]) was incubated with [γ-^{32}P]ATP and 4E-BP (lanes 1 to 4 and lanes 9 to 12) or recombinant Ypk2 (lanes 5 to 8 and lanes 13 to 16) at 30°C for 60 min and stopped with sample buffer, and proteins were separated by SDS-PAGE. The results of Coomassie staining (left) and autoradiography (kinase assay) (right) are shown. * and **, migration of heavy and light chains of the immunoprecipitation antibody. C. Tor2 phosphorylates Ypk2 at the turn motif and the hydrophobic motifs. In vitro kinase assays were carried out using the wild type (WT; lanes 1, 2, and 4) and the turn- and hydrophobic-motif mutants (S641A T659A, S641A, and T659A; lanes 3, 5, and 6, respectively) of Ypk2.

six-amino-acid residue sequence (^{237}TFDVT^{243}R in Ypk2) nearly completely conserved between these three sequences (Fig. 1B). To examine the function of this sequence we individually mutated each amino acid in this sequence to an alanine in the context of otherwise full-length, wild-type Ypk2. We then expressed these constructs in a multicopy vector under the control of the *YPK2* promoter and asked whether or not these alleles could suppress *tor2* phenotypes (Fig. 1C). The ability to suppress a *tor2* mutation (YYK241) was conferred upon full-length Ypk2 by substitution of F238 or D239 (or 240V, albeit weakly) to alanine. We provide further evidence (below) that this region does indeed inhibit the kinase activity of Ypk2. There is a similar sequence around F213 (F^{213}DNSI^{217}) in Ypk2 (Fig. 1B); however, when we replaced F213 with an alanine, we did not observe suppression (data not shown).

The TORC2 signaling pathway plays an important role in regulating polarization of the actin cytoskeleton via the Rho1/Pkc1/MAPK (cell integrity) cascade. We therefore wished to examine the effect of expression of the *YPK2*^{D239A} allele has on the cell integrity cascade. Compared to *TOR2* cell results, phosphorylation of Mpk1, the MAPK in this cascade, was diminished in *tor2* mutants (YYK241), confirming that the Rho1/Pkc1/Mpk1 cell integrity pathway is compromised in *tor2* cells (Fig. 2A). Overexpression of *YPK2*^{D239A} fully rescued Mpk1 phosphorylation, while the wild-type allele rescued poorly. YYK241 cells also showed a cell lysis phenotype, presumably because of inactivation of the Pkc1/Mpk1 cascade resulting in fragility of the cell wall. Expression of *YPK2*^{D239A} suppressed this phenotype (data not shown). We further asked whether our multicopy *YPK2* alleles were able to suppress the actin polarization defect of *tor2* cells. At 37°C 90% of *TOR2* cells displayed normal actin localization. In contrast, in *tor2-21* cells, actin patches were most often not restricted to the small bud but were observed in both the mother cell and the bud (only 31% of cells displayed normal action organization; Fig. 2B). Overexpression of *YPK2*^{D239A} and *YPK2* in *tor2-21* cells restored proper actin organization in 82% and 56% of cells, respectively (Fig. 2B and data not shown).

Given that overexpression of alleles of *YPK2* can suppress *tor2* phenotypes and that the phosphorylation of two other AGC kinases is regulated by TOR (Gad8 by Tor1 in *Schizosaccharomyces pombe* and S6K1 by mTOR in mammals), we hypothesized that Ypk2 may be directly phosphorylated by Tor2. Gad8 and S6K1 are phosphorylated in a TOR-dependent manner on serine and threonine residues residing in a so-called hydrophobic motif and the turn motif (T659 and S641 in Ypk2, respectively). TOR-dependent phosphorylation of

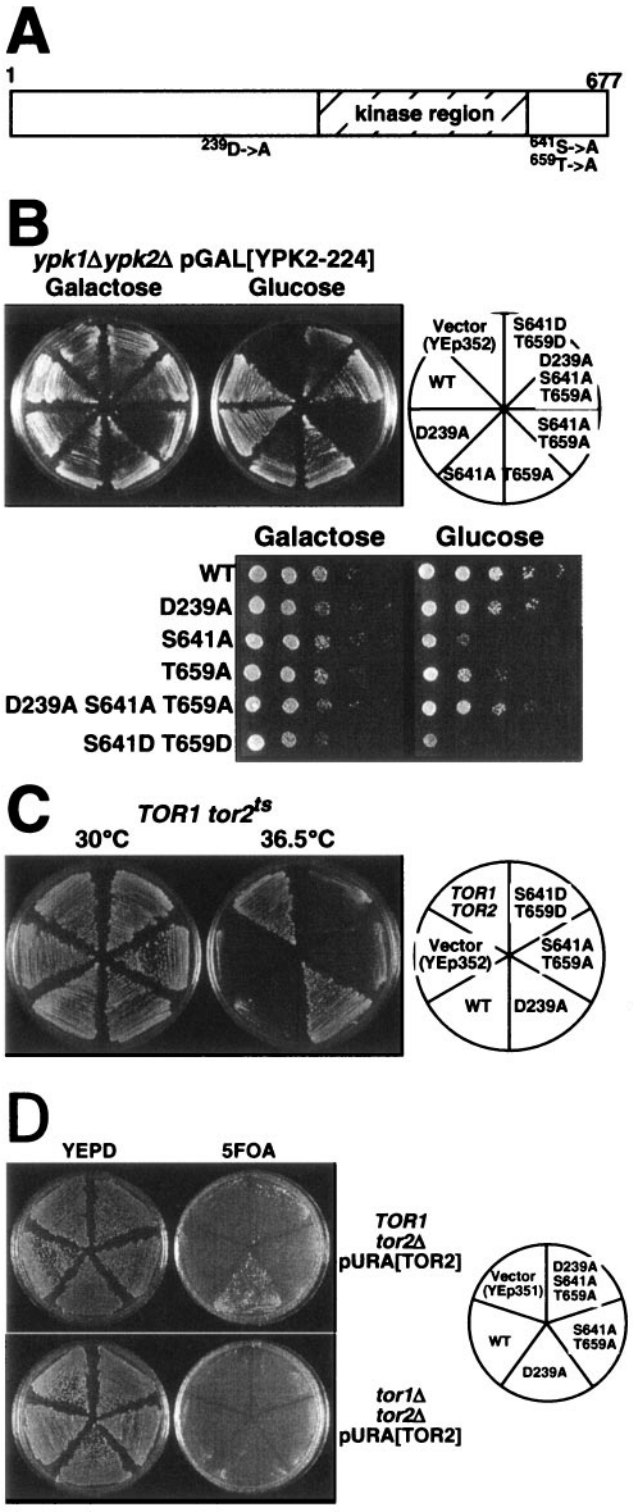

FIG. 4. *YPK2*^{D239A} overcomes mutation in *cis*, Tor2-dependent phosphorylation sites. A. Mutation sites of Ypk2. D239 is in a "TOS-like" conserved region, and S641 and T659 are in the turn motif and the hydrophobic motif (sites phosphorylated by Tor2), respectively. B. (Top) *YPK2* genes (cloned into YEp352) harboring mutation at D239 or/and Tor2-phosphorylation sites as indicated (right) were transformed into YYK373 (ypksΔ p415GAL1[*YPK2*-224]). The transformants were grown on YEP galactose (Ypk2-224 expressed; left) or YEPD (Ypk2-224 not expressed; center) plates for 2 days at 30°C.

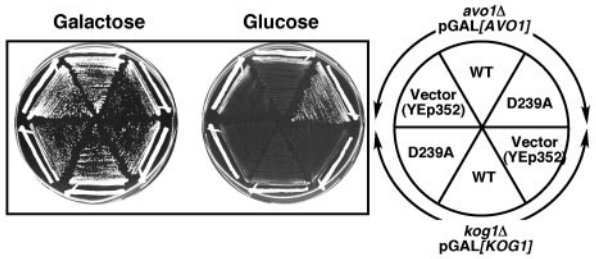

FIG. 5. *YPK2*^{D239A} suppresses growth defects associated with TORC2 disruption but not TORC1 disruption. GAL1 promoter-*KOG1* (a component of TORC1; RL93a)- and GAL1 promoter-*AVO1* (a component of TORC2; RL23-1c)-containing cells harboring *YPK2* plasmids as indicated (right) were grown in SCgalactose glycerol-Ura (left) or SCglucose-Ura (center).

these residues is indispensable for the kinase function of Gad8 and S6K1 (16, 27). Therefore, we examined whether Tor2 directly phosphorylates these C-terminal residues in Ypk2. Specifically, we assayed whether immunoprecipitated N-terminally hemagglutinin (HA)-tagged Tor2 protein (^{HA}Tor2) (Fig. 3A) could phosphorylate recombinant Ypk2 in vitro (Fig. 3B). ^{HA}Tor2 indeed phosphorylated Ypk2 (lane 13) as well as 4E-BP (a substrate of mTOR and positive control in this experiment) (lane 9), suggesting that Tor2 directly phosphorylates Ypk2. Immunoprecipitated ^{HA}Tor2^{D2298E} (kinase dead mutant) displayed no detectable kinase activity toward 4E-BP or Ypk2 (lanes 11 and 15), excluding the possibility that a co-immunoprecipitated protein kinase phosphorylates Ypk2. As shown in Fig. 3C, the turn- and hydrophobic-motif mutant (Ypk2$^{S641A\ T659A}$) was only marginally phosphorylated compared to wild-type Ypk2. We also tested Ypk2^{S641A} and Ypk2^{T659A} mutants as substrates. Ypk2^{S641A} was phosphorylated (but significantly more weakly than Ypk2^{WT}) by Tor2, while phosphorylation of Ypk2^{T659A} was largely diminished (Fig. 3C). These results indicate that Tor2 directly phosphorylates Ypk2 at S641 in the turn motif and T659 in the hydrophobic motif.

In contrast to wild-type *YPK2*, expression of *YPK2*$^{S641A\ T659A}$ failed to restore growth to a strain lacking *YPK1* and *YPK2* (*ypk*Δ), suggesting that phosphorylation of the turn and hydrophobic motifs is essential for Ypk function (Fig. 4B, top panel). The *YPK2*^{S641A} and *YPK2*^{T659A} alleles restored slow growth to *ypk*Δ cells (Fig. 4B, bottom panel). In contrast, the *YPK2*^{D239A} mutant allele could fully complement this *ypk*Δ strain, and moreover the *YPK2*$^{D239A\ S641A\ T659A}$ (combination of suppressor allele

(Bottom) Serial dilutions of the indicated transformants were spotted on SCgalactose-URA (left) and SCglucose-URA (right) and grown for 2 days at 30°C. C. A temperature-sensitive *tor2-21* (SH121 [*TOR1 tor2-21*]) mutant was transformed with YEp352-based *YPK2* plasmids as indicated (right) and grown on YEPD for 2 days at 30°C (left) or 36.5°C (center). Wild-type control cells (SH100 [*TOR1 TOR2*]) are also shown. D. *YPK2* mutant genes were transformed into YYK353 (*TOR1 tor2*Δ pRS316[*TOR2*]) (top panel) and YYK357 (*tor1*Δ *tor2*Δ pRS316[*TOR2*]) (bottom panel) as indicated in the right panel, and the resultant transformants were grown on YEPD (left) and 5-fluoroorotic acid (5FOA) plate (center) for 2 days at 30°C. On 5FOA plates, cells must lose the pRS316[*TOR2*] (harboring *URA3* marker) plasmid to grow (19).

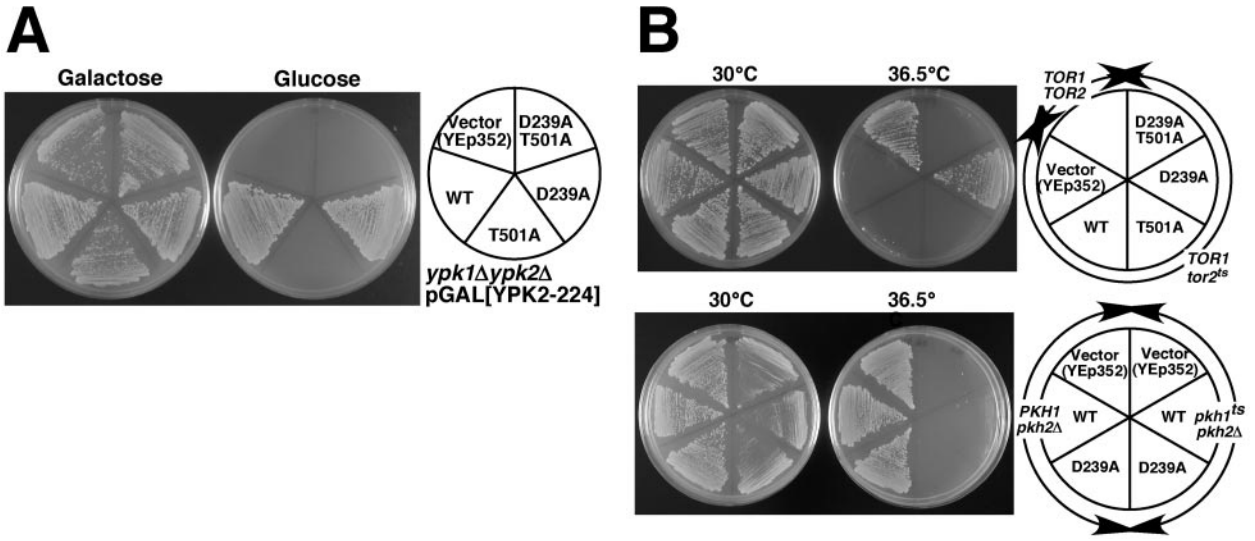

FIG. 6. *YPK2*^{D239A} acts as a *tor2*-specific suppressor. A. (Top) *YPK2* alleles (cloned into YEp352) harboring mutations at D239 or/and T501 (Pkh phosphorylation site) were transformed into YYK373 (ypksΔ p415GAL1[*YPK2*-224]). The transformants were grown on YEPgalactose (Ypk2-224 expressed; left) or YEPD (Ypk2-224 not expressed; center) plates for 2 days at 30°C. (Bottom) A temperature-sensitive *tor2-21* mutant (SH121 [*TOR1 tor2-21*]) was transformed with YEp352-based *YPK2* plasmids as indicated (right) and grown on YEPD for 2 days at 30°C (left) or 36.5°C (center). Wild-type control cells (SH100 [*TOR1 TOR2*]) are also shown. B. Temperature-sensitive *pkh* mutant cells (INA106-3B [*pkh1*^{D398G} *pkh2*Δ]) and wild-type control cells (INA28-1B [*PKH1 pkh2*Δ]) were transformed with YEp352-based *YPK2* plasmids as indicated (right) and grown on YEPD for 2 days at 30°C (left) or 36.5°C (center).

with phosphorylation mutations) allele could also complement (Fig. 4B). Overexpression of *TOR2* could not suppress the growth defect of *ypk*Δ cells, confirming that Ypks act downstream of Tor2 (data not shown).

Next, we tested the ability of a *YPK2*$^{S641D\ T659D}$ mutant to restore growth to *ypk*Δ cells. Mutation of these residues to aspartate was expected to mimic phosphorylation. *YPK2*$^{S641D\ T659D}$ restored very slow growth to *ypk*Δ cells (Fig. 4B, bottom panel). However, it did not rescue the temperature-sensitive growth of *tor2-21* cells (Fig. 4C). This suggests that these mutations cannot fully substitute for phosphoserine/threonine in the activation of Ypk2. From studies whose results were consistent with these, Matsuo et al. (27) reported that gad8-S527D/S546D (a mutation equivalent to *YPK2*$^{S641D\ T659D}$) only partially rescued the mating defect of a *gad8*Δ mutant, and Casamayor et al. (3) reported that Ypk1$^{T504D\ T662D}$ displayed no detectable activity.

Tor2 functions in two distinct complexes; we therefore wanted to determine which Tor2 complex regulates Ypk. Overexpression of *YPK2*^{D239A} or *YPK2*$^{D239A\ S641A\ T659A}$ suppressed the lethality of a *tor2* null mutant but not a *tor1*Δ *tor2*Δ double mutant, suggesting that Ypk2^{D239A} could compensate for the essential function of Tor2 but not of both Tor1 and Tor2 (Fig. 4C). Furthermore, overexpression of *YPK2*^{D239A} suppressed the growth defects of an *avo1* (TORC2) mutant but not a *kog1* (TORC1) mutant (Fig. 5). Together these results suggest that Ypks function downstream of TORC2 and not downstream of TORC1.

It is known that Ypks are downstream of Pkh1 and Pkh2, the yeast orthologues of the PDK1 protein kinase (15). Pkhs act as effectors of sphingoid-base signaling, playing important roles in cell integrity, endocytosis, actin organization, and activation of Pkc1 and Ypk2 (15). Phosphorylation of T501 in Ypk2 by Pkhs is indispensable for Ypk2 function, because *YPK2*^{T501A} or *YPK2*$^{D239A\ T501A}$ did not complement the lethality of *ypk*Δ cells (Fig. 6A, top panel). *YPK2*$^{D239A\ T501A}$ did not suppress *tor2*^{ts} cells, either (Fig. 6A, bottom panel). We also tested whether the *tor2*-suppressing *YPK2*^{D239A} allele could suppress *pkh* phenotypes. Neither *YPK2*^{WT} nor *YPK2*^{D239A} suppressed the lethality of *pkh1*^{ts} cells at the nonpermissive temperature (Fig. 6B). At a semipermissive temperature (35°C) both *YPK2* alleles weakly suppressed temperature sensitivity (data not shown). One interpretation of these results is that the Pkhs have other essential targets in addition to the Ypks (Pkc1, for example).

Lastly, we assayed the kinase activity of C-terminally HA-tagged Ypk2 immunopurified from *TOR2* or *tor2-21* cells by using a peptide (2× crosstide) as a substrate (3) (Fig. 7A). The activity of Ypk2^{WT} was significantly reduced when isolated from *tor2-21* cells compared to wild-type cell results (decreased to 2% of wild type; Fig. 7A, lanes 1 and 6), confirming that Ypk kinase activity is regulated by *TOR2*. We also found that Ypk2^{D239A} was hyperactive (sevenfold activation compared to Ypk2^{WT}; lane 3), and this activity was independent of *TOR2* (lane 8). In contrast Ypk2$^{S641A\ T659A}$ possessed a greatly reduced kinase activity (about 10% of Ypk2^{WT}; lanes 4 and 9), which was partially restored (up to 65% of Ypk2^{WT}) by introduction of the D239A mutation (lanes 5 and 10). As a control, we also performed in vitro protein kinase assays with a kinase dead Ypk2 mutant (Ypk2^{K373A}). The protein kinase activity of Ypk2^{K373A} and Ypk2$^{D239A\ K373A}$ was essentially lost, confirming that the protein kinase activity detected was an intrinsic property of Ypk2 (Fig. 7B). These results correspond well with the above-presented conclusions that Ypk activity and function are regulated by Tor2 via direct phosphorylation of S641 and

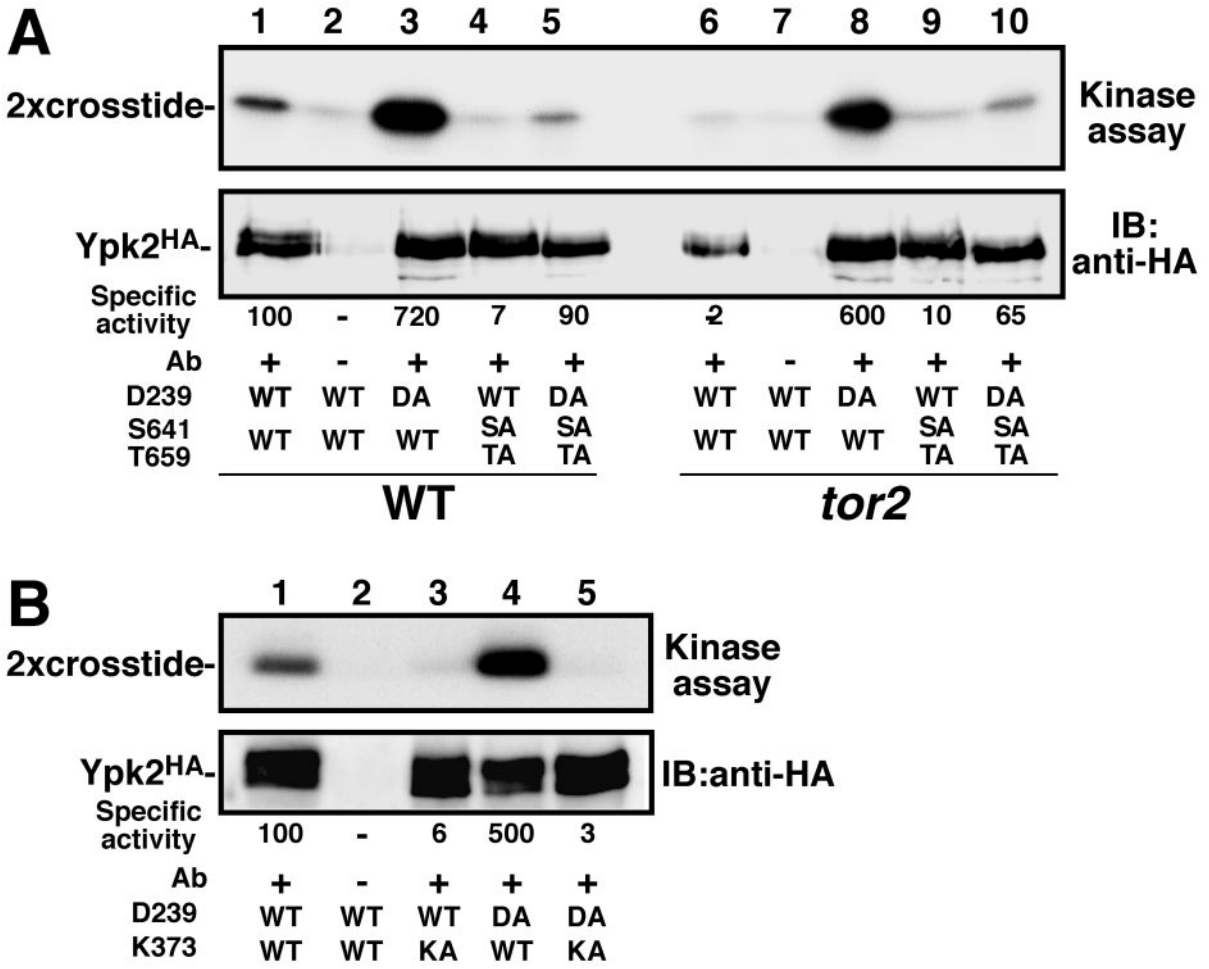

FIG. 7. Protein kinase activity of Ypk2 is regulated by *TOR2*. A. Wild-type (SH100) and *tor2-21* mutant (SH121) cells harboring YEp352-based HA-tagged *YPK2* plasmids as indicated were grown in YEPD at 30°C. $Ypk2^{HA}$ was immunoprecipitated from cell lysates and subjected to in vitro kinase assays (Kinase assay; top panel). Immunoprecipitated $Ypk2^{HA}$ was monitored by immunoblot (IB; bottom). Radioactivity and intensity of immunoblot signal were measured by BAS2000 and LAS1000 (Fuji Film), respectively, and specific activity of each sample was calculated (shown in the figure). B. Ypk2 kinase activity was assessed as described above. A kinase dead Ypk2 mutant (K373A) displayed no detectable protein kinase activity (lanes 3 and 5).

T659 and that the D239A substitution potentially disrupts an inhibitory region in Ypk and thereby increases basal activity to a level which is sufficient to compensate for the loss of activating signals from Tor2.

DISCUSSION

We have shown that Ypk2 is a direct substrate of Tor2 and likely TORC2. This observation is consistent with the recent report of Matsuo et al. (27) that the fission yeast orthologue of Ypk1/2, Gad8, is also downstream of Tor in this organism. However, whether Gad8 is directly phosphorylated by Tor has not been elucidated. Both Ypk and TORC2 have been shown to function upstream of Rho1 to mediate actin polarization and endocytosis (6, 7, 34). Thus, Ypk appears to be a missing link between TORC2 and Rho1 activation. Furthermore, given that $YPK2^{D239A}$ could suppress the temperature sensitivity and actin defects of *tor2-21* mutants, even when it is integrated in the genome (data not shown), and that it suppressed the growth defect of *tor2*Δ cells, suggests that the Ypks are the essential target of TORC2. How Ypk signaling regulates Rho1 activation remains to be determined. Recently, Audhya et al. reported that the essential homologous protein pair, Slm1 and Slm2, are also directly phosphorylated by TORC2 (1). Like Ypks, the Slms are required for polarization of the actin cytoskeleton and seem to operate upstream of the cell integrity pathway. The functions of the Slms are not known, and the precise biochemical connection between the Slms and Ypks remains to be determined. Our results suggest that Ypks that act downstream of TORC2 but not TORC1-$YPK2^{D239A}$ could not suppress the growth defects resulting from the loss of TORC1 function. However, Gelperin et al. reported that *ypk* mutant cells are rapamycin hypersensitive (9). Also, rapamycin treatment appears to enhance Mpk1 activity (24). These findings are curious, because TORC2, unlike TORC1, is rapamycin insensitive. Cross talk between TORC1 and TORC2 could provide one explanation for these observations.

We also found that *YPK2*^{D239A} has TOR2-independent kinase activity and function but is still dependent upon *PKH* genes, encoding counterparts of mammalian PDK. PDK directly phosphorylates the activation loop of AGC kinases, such as S6K (mammal) and Gad8 (fission yeast). Thus, Ypks could integrate TORC2 and Pkh-mediated sphingolipid signals to regulate and coordinate actin polarization and endocytosis.

How does D239A relieve TORC2 dependency? We propose that Ypk has two regions that mediate TOR dependency, the N-terminal inhibitory region (containing D239) and the C-terminal phosphorylation domain (the turn and hydrophobic motifs). Interestingly, mutations in the former region confer TOR-independent activity, while mutations in the latter result in a loss of TOR-dependent activation. It is possible that these regions interact with each other, although this remains to be determined. Curiously, D239 of Ypk2 resides in a sequence, ^{237}TFDVT^{243}R, which resembles the TOS motif in mammalian TOR (mTOR) target proteins (32). In mammals, mTOR forms a complex with Raptor (11) (23). Raptor binds to TOS motifs and thereby recruits substrates, i.e., S6K and 4E-BP, to mTOR (29). Thus, the TOS motif plays a positive role in mTOR signaling. In yeast, the Raptor orthologue, Kog1, is a component of TORC1 (26), and it is therefore unlikely that Kog1 recognizes the "TOS-like" regions of Ypks. Moreover, the "TOS-like" region in Ypks antagonizes TORC2 signaling. Therefore, although the TOS motifs are apparently conserved among mammalian and yeast targets of TOR, the function of this sequence does not seem to be conserved. Rather, it is reasonable that the "TOS-like" region in Ypks plays an inhibitory role in regulating Ypk activity, such as the pseudosubstrate domain in protein kinase C. Our current model of Ypk activation is as follows. When inactive, the catalytic domain of Ypk is sheltered by the "TOS-like" regulatory region. Next, TORC2 directly phosphorylates inactive Ypk at the turn and hydrophobic motifs. The possibility is not fully excluded that another protein kinase(s) may phosphorylate Ypk at these sites, but we demonstrated here that its phosphorylation by Tor2 but not by any other kinase is indeed required for activation of Ypk. Phosphorylation at the turn and hydrophobic motifs by TORC2 releases the "TOS-like" regulatory region, exposing the Ypk kinase domain to phosphorylation by Pkhs. Phosphorylation in the activation loop by Pkhs results in fully active Ypk (3).

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REFERENCES

1. **Audhya, A., R. Loewith, A. B. Parsons, L. Gao, M. Tabuchi, H. Zhou, C. Boone, M. N. Hall, and S. D. Emr.** 2004. Genome-wide lethality screen identifies new PI4,5P(2) effectors that regulate the actin cytoskeleton. EMBO J. **23:**3747–3757.
2. **Barbet, N. C., U. Schneider, S. B. Helliwell, I. Stansfield, M. F. Tuite, and M. N. Hall.** 1996. TOR controls translation initiation and early G_1 progression in yeast. Mol. Biol. Cell **7:**25–42.
3. **Casamayor, A., P. D. Torrance, T. Kobayashi, J. Thorner, and D. R. Alessi.** 1999. Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. Curr. Biol. **9:**186–197.
4. **Chen, P., K. S. Lee, and D. E. Levin.** 1993. A pair of putative protein kinase genes (YPK1 and YPK2) is required for cell growth in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **236:**443–447.
5. **Cherkasova, V. A., and A. G. Hinnebusch.** 2003. Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. Genes Dev. **17:**859–872.
6. **deHart, A. K., J. D. Schnell, D. A. Allen, and L. Hicke.** 2002. The conserved Pkh-Ypk kinase cascade is required for endocytosis in yeast. J. Cell Biol. **156:**241–248.
7. **deHart, A. K., J. D. Schnell, D. A. Allen, J. Y. Tsai, and L. Hicke.** 2003. Receptor internalization in yeast requires the Tor2-Rho1 signaling pathway. Mol. Biol. Cell **14:**4676–4684.
8. **Friant, S., R. Lombardi, T. Schmelzle, M. N. Hall, and H. Riezman.** 2001. Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. EMBO J. **20:**6783–6792.
9. **Gelperin, D., L. Horton, A. DeChant, J. Hensold, and S. K. Lemmon.** 2002. Loss of ypk1 function causes rapamycin sensitivity, inhibition of translation initiation and synthetic lethality in 14-3-3-deficient yeast. Genetics **161:**1453–1464.
10. **Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. Andre, A. P. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, D. E. Kelly, S. L. Kelly, P. Kotter, D. LaBonte, D. C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S. L. Ooi, J. L. Revuelta, C. J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D. D. Shoemaker, S. Sookhai-Mahadeo, R. K. Storms, J. N. Strathern, G. Valle, M. Voet, G. Volckaert, C. Y. Wang, T. R. Ward, J. Wilhelmy, E. A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippsen, R. W. Davis, and M. Johnston.** 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. Nature **418:**387–391.
11. **Hara, K., Y. Maruki, X. Long, K. Yoshino, N. Oshiro, S. Hidayat, C. Tokunaga, J. Avruch, and K. Yonezawa.** 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell **110:**177–189.
12. **Harris, T. E., and J. C. Lawrence, Jr.** 2003. TOR signaling. Sci. STKE **2003:**re15.
13. **Helliwell, S. B., I. Howald, N. Barbet, and M. N. Hall.** 1998. TOR2 is part of two related signaling pathways coordinating cell growth in *Saccharomyces cerevisiae*. Genetics **148:**99–112.
14. **Hilti, N., D. Baumann, A. M. Schweingruber, P. Bigler, and M. E. Schweingruber.** 1999. Gene ste20 controls amiloride sensitivity and fertility in *Schizosaccharomyces pombe*. Curr. Genet. **35:**585–592.
15. **Inagaki, M., T. Schmelzle, K. Yamaguchi, K. Irie, M. N. Hall, and K. Matsumoto.** 1999. PDK1 homologs activate the Pkc1-mitogen-activated protein kinase pathway in yeast. Mol. Cell. Biol. **19:**8344–8352.
16. **Isotani, S., K. Hara, C. Tokunaga, H. Inoue, J. Avruch, and K. Yonezawa.** 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. J. Biol. Chem. **274:**34493–34498.
17. **Jacinto, E., and M. N. Hall.** 2003. Tor signalling in bugs, brain and brawn. Nat. Rev. Mol. Cell Biol. **4:**117–126.
18. **Jiang, Y., and J. R. Broach.** 1999. Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. EMBO J. **18:**2782–2792.
19. **Kaiser, C., S. Michaelis, A. Mitchell, and Cold Spring Harbor Laboratory.** 1994. Methods in yeast genetics: a Cold Spring Harbor laboratory course manual, 1994 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Kamada, Y., T. Funakoshi, T. Shintani, K. Nagano, M. Ohsumi, and Y. Ohsumi.** 2000. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J. Cell Biol. **150:**1507–1513.
21. **Kamada, Y., U. S. Jung, J. Piotrowski, and D. E. Levin.** 1995. The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. Genes Dev. **9:**1559–1571.
22. **Kawai, M., A. Nakashima, M. Ueno, T. Ushimaru, K. Aiba, H. Doi, and M. Uritani.** 2001. Fission yeast tor1 functions in response to various stresses including nitrogen starvation, high osmolarity, and high temperature. Curr. Genet. **39:**166–174.
23. **Kim, D. H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini.** 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell **110:**163–175.
24. **Krause, S. A., and J. V. Gray.** 2002. The protein kinase C pathway is required for viability in quiescence in *Saccharomyces cerevisiae*. Curr. Biol. **12:**588–593.
25. **Kubota, H., T. Obata, K. Ota, T. Sasaki, and T. Ito.** 2003. Rapamycin-induced translational derepression of GCN4 mRNA involves a novel mech-

anism for activation of the eIF2 alpha kinase GCN2. J. Biol. Chem. **278:** 20457–20460.

26. **Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J. L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe, and M. N. Hall.** 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell **10:**457–468.
27. **Matsuo, T., Y. Kubo, Y. Watanabe, and M. Yamamoto.** 2003. *Schizosaccharomyces pombe* AGC family kinase Gad8p forms a conserved signaling module with TOR and PDK1-like kinases. EMBO J. **22:**3073–3083.
28. **Mizushima, N., A. Yamamoto, M. Matsui, T. Yoshimori, and Y. Ohsumi.** 2004. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol. Biol. Cell **15:**1101–1111.
29. **Nojima, H., C. Tokunaga, S. Eguchi, N. Oshiro, S. Hidayat, K. Yoshino, K. Hara, N. Tanaka, J. Avruch, and K. Yonezawa.** 2003. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J. Biol. Chem. **278:**15461–15464.
30. **Powers, T., and P. Walter.** 1999. Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in *Saccharomyces cerevisiae*. Mol. Biol. Cell **10:**987–1000.
31. **Roelants, F. M., P. D. Torrance, N. Bezman, and J. Thorner.** 2002. Pkh1 and pkh2 differentially phosphorylate and activate ypk1 and ykr2 and define protein kinase modules required for maintenance of cell wall integrity. Mol. Biol. Cell **13:**3005–3028.
32. **Schalm, S. S., and J. Blenis.** 2002. Identification of a conserved motif required for mTOR signaling. Curr. Biol. **12:**632–639.
33. **Schmelzle, T., S. B. Helliwell, and M. N. Hall.** 2002. Yeast protein kinases and the RHO1 exchange factor TUS1 are novel components of the cell integrity pathway in yeast. Mol. Cell. Biol. **22:**1329–1339.
34. **Schmidt, A., M. Bickle, T. Beck, and M. N. Hall.** 1997. The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. Cell **88:**531–542.
35. **Schmidt, A., J. Kunz, and M. N. Hall.** 1996. TOR2 is required for organization of the actin cytoskeleton in yeast. Proc. Natl. Acad. Sci. USA **93:** 13780–13785.
36. **Sun, Y., R. Taniguchi, D. Tanoue, T. Yamaji, H. Takematsu, K. Mori, T. Fujita, T. Kawasaki, and Y. Kozutsumi.** 2000. Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. Mol. Cell. Biol. **20:**4411–4419.
37. **Weisman, R., and M. Choder.** 2001. The fission yeast TOR homolog, $tor1^{+}$, is required for the response to starvation and other stresses via a conserved serine. J. Biol. Chem. **276:**7027–7032.
38. **Wilkinson, M. G., T. S. Pino, S. Tournier, V. Buck, H. Martin, J. Christiansen, D. G. Wilkinson, and J. B. Millar.** 1999. Sin1: an evolutionarily conserved component of the eukaryotic SAPK pathway. EMBO J. **18:**4210–4221.