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Loss of Ypk1 Function Causes Rapamycin Sensitivity, Inhibition of Translation Initiation and Synthetic Lethality in 14-3-3-Deficient Yeast

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ABSTRACT

14-3-3 proteins bind to phosphorylated proteins and regulate a variety of cellular activities as effectors of serine/threonine phosphorylation. To define processes requiring 14-3-3 function in yeast, mutants with increased sensitivity to reduced 14-3-3 protein levels were identified by synthetic lethal screening. One mutation was found to be allelic to *YPK1*, which encodes a Ser/Thr protein kinase. Loss of Ypk function causes hypersensitivity to rapamycin, similar to 14-3-3 mutations and other mutations affecting the TOR signaling pathway in yeast. Similar to treatment with rapamycin, loss of Ypk function disrupted translation, at least in part by causing depletion of eIF4G, a central adaptor protein required for cap-dependent mRNA translation initiation. In addition, Ypk1 as well as eIF4G protein levels were rapidly depleted upon nitrogen starvation, but not during glucose starvation, even though both conditions inhibit translation initiation. These results suggest that Ypk regulates translation initiation in response to nutrient signals, either through the TOR pathway or in a functionally related pathway parallel to TOR.

THE 14-3-3 proteins are a highly conserved family of abundant ~30-kD proteins found in all eukaryotes (Fu *et al.* 2000). They bind to target proteins upon phosphorylation within a 14-3-3 binding motif and serve as effectors of Ser/Thr phosphorylation (Muslin *et al.* 1996; Yaffe *et al.* 1997; Fu *et al.* 2000; Yaffe and Elia 2001). 14-3-3 proteins are best known for their roles in signal transduction pathways, including those regulating cell cycle and checkpoint control, cell survival, and growth (see Yaffe and Cantley 1999; Baldin 2000; Fu *et al.* 2000 and references therein). However, 14-3-3's have been implicated in a wide variety of other processes, such as regulation of ADP ribosylation of small GTPases (Fu *et al.* 1993), nitrate reductase (Moorhead *et al.* 1996), neurotransmitter biosynthesis (Ichimura *et al.* 1987), the cytoskeleton (Liao and Omary 1996), secretion (Morgan and Burgoyne 1992; Skoulakis and Davis 1996; Roth *et al.* 1999), and mitochondrial protein import (Alam *et al.* 1994).

Saccharomyces cerevisiae has two 14-3-3 isoforms, encoded by *BMH1* and *BMH2* (Gelperin *et al.* 1995; van Heusden *et al.* 1995). The proteins are 92% identical, although Bmh1p is the predominant form, accounting for ≥75% of 14-3-3 in a yeast cell (Garrels *et al.* 1994; Gelperin *et al.* 1995). Deletion of either *BMH1* or *BMH2* alone does not affect cell growth, but the double deletion is lethal in most strain backgrounds (Gelperin *et al.* 1995; van Heusden *et al.* 1995; Roberts *et al.* 1997).

In budding yeast, 14-3-3's have been implicated in a number of processes as well (see van Hemert *et al.* 2001 for review), although the direct target(s) of 14-3-3 proteins in many of these pathways is still not known. One of the known specific roles of yeast 14-3-3's is as downstream effectors in the rapamycin-sensitive *t*arget *o*f *r*apamycin (TOR) pathway (Bertram *et al.* 1998; Beck and Hall 1999). TOR is a Ser/Thr kinase that plays a central role in the integration of nutrient status inputs with growth control in yeast, as well as mammalian cells (Schmelzle and Hall 2000; Gingras *et al.* 2001; Raught *et al.* 2001). Treatment of yeast cells with rapamycin leads to a growth arrest resembling that in starved cells or cells entering stationary phase (Barbet *et al.* 1996). Associated with this is a rapid inhibition of protein synthesis (Barbet *et al.* 1996), one of the most energy-consuming processes in the cell (Warner 1999), and a concomitant inhibition of tRNA and ribosomal biogenesis (Zaragoza *et al.* 1998; Cardenas *et al.* 1999; Hardwick *et al.* 1999; Powers and Walter 1999). In addition, rapamycin induces autophagy (Noda and Ohsumi 1998; Kamada *et al.* 2000) and transcription of starvation and stress response genes (Barbet *et al.* 1996; Beck and Hall 1999; Cardenas *et al.* 1999; Hardwick *et al.* 1999), and it affects the turnover of nutrient permeases (Schmidt *et al.* 1998; Beck *et al.* 1999). TOR is encoded by two genes in yeast, *TOR1* and *TOR2* (Kunz *et al.* 1993; Helliwell *et al.* 1994). Loss of TOR function resembles the phenotype of cells treated with rapamycin, although TOR2 also has a second rapamycin-

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TABLE 1

Yeast strains used in this study

Strain	Genotype	Plasmid	Source
SL1320	*MAT*α *bmh2-*Δ*::URA3 leu2 ura3-52 trp1 his3-*Δ*200 GAL2*		Gelperin *et al.* (1995)
SL1386	*MAT*α *bmh1-*Δ*::LEU2 leu2 ura3-52 trp1 his3-*Δ*200 GAL2*		Gelperin *et al.* (1995)
SL1388	*MAT***a** *bmh1-*Δ*::LEU2 leu2 ura3-52 trp1 his3-*Δ*200 GAL2*		Gelperin *et al.* (1995)
SL1462	*MAT***a** *leu2 ura3-52 trp1 his3-*Δ*200 GAL2*		Gelperin *et al.* (1995)
SL1463	*MAT*α *leu2 ura3-52 trp1 his3-*Δ*200 GAL2*		Gelperin *et al.* (1995)
SL1528	*MAT***a**/α *leu2/leu2 ura3-52/ura3-52 trp1/trp1 his3-200/his3-*Δ*200 GAL2/GAL2*		Gelperin *et al.* (1995)
SL2067	*MAT*α *bmh2-*Δ*::HIS3 leu2-*Δ*1 ura3-52 trp1-*Δ*1 his3-*Δ*200 ade2-101*		This study
SL2136	*MAT***a** *bmh2-*Δ*::HIS3 leu2-*Δ*1 ura3-52 trp1-*Δ*1 his3-*Δ*200 ade2-101 lys2-801*	pDG46	This study
SL2331	*MAT*α *bmh2-*Δ*::HIS3 ypk1-2(bms3-1) leu2 ura3-52 trp1 his3-*Δ*200 ade2-101 lys2-801*	pDG46	This study
SL2334	*MAT*α *bmh2-*Δ*::HIS3 ypk1-2(bms3-1) leu2 ura3-52 trp1 his3-*Δ*200 ade2-101 lys2-801*		This study
SL2545	*MAT*α *ypk1-*Δ*::TRP1 ura3-52 leu2 trp1 his3-*Δ*200*		This study
SL2631	*MAT***a** *bmh1-*Δ*::LEU2 leu2 ura3 trp1 HIS3 ade2 ade3*	pDG56	This study
SL2647	*MAT*α *bmh1-*Δ*::LEU2 leu2 ura3 trp1 his3 ade2 ade3 lys2*		This study
SL2830	MATα *bmh1-*Δ*::LEU2 bmh2-*Δ*::URA3 leu2 ura3-52 trp1 his3 ade2 ade3*	pDG56	This study
SL3246	*MAT***a** *bms1-1 leu2 ura3-52 trp1 ade2 ade3 his3-*Δ*200*		This study
SL3365	*MAT*α *ypk1-2 ura3-52 leu2 trp1 his3-*Δ*200*		This study
SL4235	*MAT***a** *ypk1-*Δ*::TRP1 leu2 ura3-52 trp1 his3-*Δ*200*		This study
AC301	*MAT***a** *pkh1-*Δ*::TRP1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ssd1-*Δ*2*		Casamayor *et al.* (1999)
AC303	*MAT***a** *pkh2-*Δ*::HIS3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ssd1-*Δ*2*		Casamayor *et al.* (1999)
MH281	*MAT***a** *leu2-3,112 ura3-52 rme1 trp1 his4 HML***a**		Helliwell *et al.* (1994)
MH580	*MAT***a** *tor1-*Δ*::LEU2 leu2-3,112 ura3-52 rme1 trp1 his4 HML***a**		Helliwell *et al.* (1994)
YES1[a]	*MAT***a** *ypk2-*Δ*::TRP1(=ykr2-*Δ*1::TRP1) ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52*		Casamayor *et al.* (1999)
YPT40[a]	*MAT***a** *ypk1-1*^{ts}*:HIS3 ypk2-*Δ*::TRP1(=ykr2-*Δ*1::TRP1) ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52*		Casamayor *et al.* (1999)

[a] *YPK2* is also referred to as *YKR2* in some studies (e.g., Kubo *et al.* 1989; Casamayor *et al.* 1999).

independent essential function (Kunz *et al.* 1993; Helliwell *et al.* 1994; Zheng *et al.* 1995; Barbet *et al.* 1996; Schmidt *et al.* 1996). The 14-3-3's function downstream of TOR by binding and retaining the stress-responsive transcription factors Msn2p and Msn4p in the cytoplasm (Beck and Hall 1999). Upon rapamycin treatment Msn2 and -4p dissociate from 14-3-3's and are released into the nucleus (Beck and Hall 1999).

To uncover pathways and factors regulated by 14-3-3 proteins in yeast we sought mutations in genes that cause sensitivity to reduced 14-3-3 levels by the synthetic lethal screening technique. This screen identified a hypomorphic allele of *YPK1*, which encodes a Ser/Thr protein kinase. Here we show that loss of Ypk function leads to hypersensitivity to rapamycin and inhibition of translation initiation. Further analysis suggests that Ypk1p may play a role upstream of TOR or in a functionally overlapping pathway parallel to TOR.

MATERIALS AND METHODS

Strains used and genetic methods: Strains used in this study are listed in Table 1. Genetic methods were performed essentially as in Guthrie and Fink (1991). Yeast extract peptone dextrose (YEPD) and synthetic selective dropout media were prepared as described in Nelson and Lemmon (1993). YEPD + 3% formamide medium and other sensitivity media were prepared as described in Hampsey (1997). Rapamycin was dissolved in ethanol and added to YEPD medium at indicated concentrations. Yeast transformations were performed by the method of Gietz *et al.* (1992).

Plasmid construction: Plasmids were propagated in *Escherichia coli* DH5α and are listed in Table 2. Construction of plasmids for this study was as follows: pDG45 (*BMH2*, *CEN*, *TRP1*) was created by cloning a 4.2-kb *Kpn*I-*Cla*I fragment containing *BMH2* from a genomic library plasmid into pRS314 (Sikorski and Hieter 1989). pDG46 (*BMH2*, *CEN*, *ADE2*, *URA3*) was created in two steps. First, a 2238-bp *ADE2* fragment from pASZ11 (Stotz and Linder 1990) was cloned into the *Sma*I site of pRS316 (Sikorski and Hieter 1989). Second, a 4.2-kb *Kpn*I-*Cla*I fragment containing *BMH2* was ligated into the *Kpn*I and *Cla*I sites. pDG53 (*LEU2*, *YPK1*) was generated by cloning a 3.7-kb *Bgl*II-*Xho*I *YPK1* fragment into the *Bam*HI-*Xho*I sites of the *LEU2* integrating vector, pRS305 (Sikorski and Hieter 1989). pDG54 (*YPK1*, 2μ, *URA3*) contains the 3.7-kb *Bgl*II-*Xho*I *YPK1* fragment cloned into the *Bam*HI-*Xho*I sites of pRS426 (Christianson *et al.* 1992). pDG56 (*CEN*, *ADE3*, *TRP1*, *BMH1*) was created by cloning a 5.5-kb *Bam*HI-*Sal*I fragment containing *ADE3* into pJW42 (described in Gelperin *et al.* 1995). pDG58 (*BMH2*, *CEN*, *URA3*) was created by cloning a 3721-bp *Sac*I-*Xba*I fragment containing *BMH2* into pRS316. pDG59 (*BMH1*, 2μ, *URA3*) contains a 3.2-kb

TABLE 2

Plasmids used in this study

Plasmid	Markers	Source
pAD1	2μ *YPK1-3HA:His3MX URA3*	This study
pDG45	*CEN TRP1 BMH2*	This study
pDG46	*CEN ADE2 URA3 BMH2*	This study
pDG53	*LEU2 YPK1 (YIp-YPK1)*	This study
pDG54	2μ *URA3 YPK1*	This study
pDG56	*CEN ADE3 TRP1 BMH1*	This study
pDG58	*CEN URA3 BMH2*	This study
pDG59	2μ *URA3 BMH1*	This study
pDG60	*CEN URA3 BMH1*	This study
pJK3-3	2μ *URA3 TOR2*	Kunz *et al.* (1993)
pJK12	2μ *URA3 TOR2-1*^{r}	Kunz *et al.* (1993)
pJW18	2μ *URA3 BMH2*	Gelperin *et al.* (1995)
pL272	*ypk1-Δ::TRP1*	Chen *et al.* (1993)

*Eco*RI-*Kpn*I fragment of *BMH1* cloned into pRS426. pDG60 (*BMH1*, *CEN*, *URA3*) was created by cloning a 3205-bp *Eco*RI-*Kpn*I fragment containing *BMH1* into pRS316. The plasmid for expression of Ypk1p tagged at the C terminus with a triple HA epitope (pAD1) was made by cotransformation of pDG54 (*YPK1*, 2μ, *URA3*) and a PCR product encoding the triple HA tag followed by the His3Mx6 gene and flanked by 52 bp upstream and 55 bp downstream of the stop codon of *YPK1*. The PCR product was made with primers 5′-GCAACCTT TACTACAGCTAGGTAGCTCAATGGTGCAAGGTAGAAGC ATTAGACGGATCCCCGGGTTAATTAA-3′ and 5′-CGAAAT ATAAATCCTAGAACTTAAATTGCGCCATTGGTACAGTTG CTTCATCTTGGAATTCGAGCTCGTTTAAAC using pFA6a-3HA-His3Mx6 (Longtine *et al.* 1998) as a template.

Synthetic lethal screens: Two synthetic lethal screens were carried out. The first was performed with *bmh1-Δ* using the *ade2 ade3/ade2 ADE3* red/white colony sectoring method (Bender and Pringle 1991). A *bmh1-Δ ade2 ade3* strain, which forms white colonies, was transformed with a centromeric *BMH1*, *ADE3*, *TRP1* plasmid (pDG56) to generate SL2631. Complementation of *ade3* leads to red colony formation due to the residual *ade2* mutation. However, this strain is able to lose the *BMH1* plasmid to yield red colonies with white sectors (sector$^+$). Colonies arising from mutants that have an increased requirement for *BMH1* will lose the plasmid at a reduced rate and appear sector$^-$ (mostly or completely red). SL2631 was mutagenized by exposure to UV to 10–30% viability. After 3 days of incubation at 30° in the dark, ~60,000 colonies were visually screened for a sector$^-$ phenotype. Twenty-one sector$^-$ candidates were identified after restreaking. Candidates were transformed with a *CEN*, *URA3*, *BMH1* plasmid (pDG58) and 17 candidates unable to become sector$^+$ were discarded. To identify recessive mutations, candidates were mated to SL2647 and tested for sectoring. All candidate heterozygous diploids tested sector$^+$. The resulting diploids were sporulated and dissected. One candidate did not have 2:2 segregation of sector$^-$ to sector$^+$ and was discarded. To sort the remaining three candidates into complementation groups, mutants were crossed to each other and the diploids were scored for sectoring. Each candidate was also crossed to a *bmh1-Δ bmh2-Δ* tester strain (SL2830) to identify mutations unable to complement a *bmh2-Δ* mutation. One candidate was unable to complement the *bmh1-Δ bmh2-Δ* tester strain and was presumed to be due a mutation in *BMH2*. This candidate was not studied further. The other two candidates were designated *bms1-1* and *bms2-1* for ***bmh*-s**ensitive. These mutants were then backcrossed three times to the parental strain before further characterization. *bms2-1* had a relatively weak sector$^-$ phenotype and has not been further characterized.

A second synthetic lethal screen with *bmh2-Δ* was performed using the *ade2/ADE2* sectoring method to follow plasmid loss essentially as described in White and Johnson (1997). A *bmh2-Δ ade2* strain was transformed with pDG46 carrying *BMH2* on an *ADE2*, *URA3* plasmid to yield SL2136. This strain grew as white colonies with red sectors (sector$^+$) and nonsectoring mutant candidates appeared white (sector$^-$). SL2136 was mutagenized with methane sulfonic acid ethyl ester (EMS; Guthrie and Fink 1991) to 25–30% viability and grown on YEPD plates at 30° at a density of 200–300 colonies per plate. Approximately 96,000 colonies were visually screened for a sector$^-$ phenotype. Eighteen sector$^-$ colonies that retained their sector$^-$ phenotype upon restreaking were identified. Candidates were then transformed with a *TRP1*, *BMH2*, *CEN* plasmid (pDG45) and the empty vector (pRS314) to ensure that they were able to become sector$^+$ when an independent *BMH2* plasmid was supplied. Nine candidates either remained sector$^-$ in the presence of pDG45 or became sector$^+$ in the presence of the empty *TRP1* vector and were eliminated. The nine remaining mutant candidates were mated to an *ade2 bmh2-Δ* strain of the opposite mating type (SL2067) and the diploids were tested for sectoring. Recessive mutants with a sector$^+$ phenotype after crossing were subjected to tetrad analysis. Six candidates failed to segregate as single locus mutations, one candidate was judged to be too sick to pursue further, and one was unable to sporulate as a heterozygous diploid and was discarded. The remaining candidate, *bms3-1* (*ypk1-2*, see below), was analyzed further. This candidate was backcrossed three times to the parental strain SL2136 before use in further studies.

Cloning *bmh* synthetic mutations: Cloning of the wild-type gene for *bms1-1*, which causes temperature-sensitive growth, is described elsewhere (Gelperin *et al.* 2001). The *bms3-1* mutant grew at all temperatures, but was found to grow poorly on 3% formamide when it was tested for increased sensitivity to a number of different ions and inhibitors (see Hampsey 1997 for conditions tested). Therefore, to clone the wild-type *BMS3* gene, the *bms3-1* strain, SL2331, was transformed with a YEp13-based genomic library (Matsuura and Anraku 1993) and transformants were selected directly onto C-LEU + 3% formamide plates. One library plasmid was able to rescue both the formamide sensitivity and the sector$^-$ phenotype of SL2331. Both ends of the genomic library insert were sequenced using primers flanking the insert cloning site. The minimal complementing region was identified by subcloning and retesting sectoring and formamide sensitivity in SL2331. The complementing open reading frame was found to be *YPK1*.

***YPK1* gene deletion and confirmation that *bms3-1* is allelic to *YPK1*:** *YPK1* was deleted in SL1528 using pL272 (Chen *et al.* 1993) digested with Pvu2 to generate a *ypk1-Δ::TRP1* deletion fragment. Correct integration was confirmed by Southern blotting and haploid segregants containing the *ypk1-Δ:TRP1* allele were generated by tetrad analysis.

To confirm that the *bms3-1* mutation is allelic to *YPK1*, pDG53 (YIp-*YPK1*) was linearized within *YPK1* with *Nsi*I and transformed into SL2331 [*bmh2-Δ::HIS3 bms3-1* + pDG56 (*BMH2*)]. Proper integration was confirmed by Southern blot. Resulting integrants were mated to a *bmh2-Δ BMS3* strain (SL2136) and sporulated for tetrad analysis. Spore segregants were scored for colony sectoring and for growth on YEPD + 3% formamide. No sector$^-$ or formamide-sensitive spores were found in 24 tetrads (combined data of two independent integrants), demonstrating that the mutation responsible for increased dependence on *BMH2* was in *YPK1*.

Fractionation of ribosomes: All procedures were performed at 4° except where indicated. Yeast cells from 50 ml of midlog-phase culture were pelleted, resuspended in 5 ml ice-cold 100 μg/ml cycloheximide (Calbiochem, La Jolla, CA) for 1 min, and repelleted. Lysates were made by glass bead lysis for 4 min, with intermittent cooling on ice, in 1.0 ml polysome buffer [PB; 100 mM KCl, 2 mM magnesium acetate, 20 mM HEPES (pH 7.4), 14.4 mM β-mercaptoethanol, 100 μg/ml cycloheximide]. The cell lysate was centrifuged at 5000 rpm for 8 min in a microcentrifuge and the supernatant was removed. Five to 10 A_{254} units were loaded onto a 16.2-ml 10–50% sucrose gradient containing 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.4), and 2 mM dithiothreitol and centrifuged in a Beckman SW28.1 rotor at 27,000 rpm for 4.5 hr. Gradients were collected with continuous monitoring at 254 nm using an ISCO UA-5 absorbance detector and 1640 gradient collector.

Immunoblots: To examine eIF4G stability in the *ypk-ts* (YPT40) and the control *ypk2*-Δ (YES1) strains, cells were grown to midlog phase in YEPD at 25° and a zero time sample was harvested. Then cells were washed and inoculated into fresh YEPD prewarmed to 37° at 0.25×10^7 cells/ml. At each time point before and after the shift to 37°, 1×10^8 cells were harvested and washed in dH_2O and the final cell pellet was frozen in a microcentrifuge tube at −80°. Samples were thawed and resuspended in 0.4 ml PB supplemented with 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail prepared as described previously (Stepp *et al.* 1995). Glass beads (0.4 g) were added and samples were vortexed on high for 5 × 1 min with icing in between. Extracts were centrifuged at 2700 × *g* for 8 min at 4° and the supernatant was recovered. Samples (0.1 A254 units) were separated on SDS polyacrylamide gels and proteins were transferred to nitrocellulose. Blots were stained with amido black to confirm equal protein loading, blocked in 5% milk in 1× Tris-buffered saline plus 0.1% Tween-20, and immunoblotted for indicated proteins.

For experiments examining the effect of starvation on Ypk1p, eIF4G, and eIF4E, a wild-type strain, SL1462, was transformed with pAD1 (*YPK1-HA*, 2μ) and grown to midlog phase in complete synthetic medium lacking histidine plus 2% glucose (C-HIS). A zero time cell sample was harvested. Remaining cells were washed in dH_2O and resuspended at 0.25×10^7 cells/ml in normal growth medium (C-HIS, not shown), synthetic yeast nitrogen base medium minus ammonium sulfate and amino acids plus 2% glucose (nitrogen starvation medium), or C-HIS minus glucose (glucose starvation). At each time point 1×10^8 cells were harvested and washed one time with dH_2O and pellets were frozen. For extraction, pellets were resuspended in 0.5 ml of a lysis buffer containing 50 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 150 mM NaCl, protease inhibitors (see above), and phosphatase inhibitors (50 mM NaF, 1 mM $NaVO_4$). Cells were lysed by addition of glass beads to 40% of the cell volume and by vortexing as described above. Lysates were spun at 4000 × *g* at 4° for 10 min. Extract samples (0.5 A254 units) were separated on SDS gels and prepared for immunoblotting as described above.

Primary antibodies used for immunoblots were: rabbit anti-eIF4G (1:2000) and rabbit anti-eIF4E (1:2000; gifts of Alan Sachs); mouse anti-Rpl3 monoclonal antibody (1:5000; gift of J. Warner); rabbit anti-Apm3p (1:5000; Panek *et al.* 1997); and rat anti-HA monoclonal antibody 3F10 (1:5000; Roche Molecular Biochemicals). Rabbit primary antibodies were detected with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Zymed, South San Francisco, CA); anti-Rpl3p antibody was detected with a goat anti-mouse antibody conjugated to HRP (Kirkegaard & Perry); and the anti-HA rat monoclonal was detected with HRP-conjugated rabbit anti-rat IgG (Zymed). Immunoblots were developed using enhanced chemiluminescence (Amersham). For quantification, films from exposures in the linear range were scanned and analyzed using NIH Image.

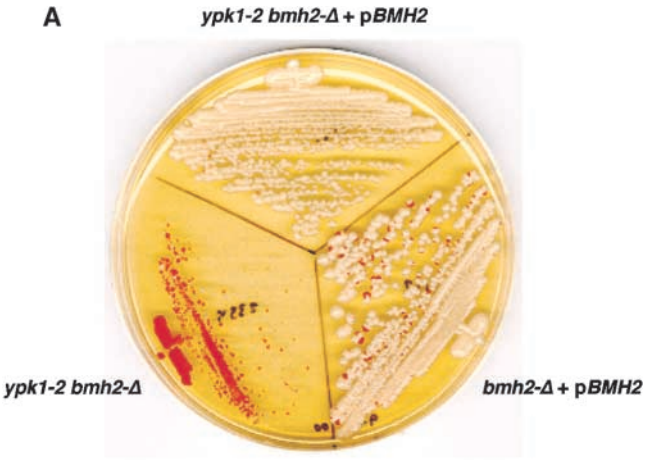
RESULTS

Identification of 14-3-3 synthetic lethal mutants and cloning of the genes: To identify pathways regulated by 14-3-3 proteins, we screened for mutants that are hypersensitive to reduced levels of 14-3-3 using a synthetic lethal approach. Starting strains were deleted for only one of the two 14-3-3 genes, and thus we screened for mutants that have impaired function in the absence of one 14-3-3 gene, even though the other one is present. Four complementation groups were identified that had an increased requirement for the presence of *BMH1* and/or *BMH2* (see materials and methods for details). One mutation from the screen for *bmh1*-Δ synthetic lethal mutants failed to complement a *bmh2*-Δ mutation, implying that the mutation was in *BMH2*. A *bmh2* mutation was expected from this screen, since *bmh1*-Δ *bmh2*-Δ cells are lethal in most genetic backgrounds (Gelperin *et al.* 1995; van Heusden *et al.* 1995).

Another mutation, *bms1-1*, causes temperature-sensitive growth on its own, with a restrictive temperature of 34°–35° (not shown). This mutation is not completely lethal in the presence of *bmh1*-Δ or *bmh2*-Δ, but the combined mutations lead to synergistic growth defects at the *bms1-1* semipermissive temperatures of 30° and 32°, with the effect of *bmh1*-Δ being more severe than that of *bmh2*-Δ (not shown). We cloned *BMS1* by complementation of its temperature-sensitive phenotype and showed that the gene corresponds to YPL217c, a previously uncharacterized open reading frame (reported in Gelperin *et al.* 2001). YPL217c/*BMS1* is an essential gene that encodes a novel GTP-binding protein of the nucleolus that is required for an early step in 40S ribosomal subunit biogenesis (Gelperin *et al.* 2001; Wegierski *et al.* 2001). The mutant in the third complementation group, *bms2-1*, had a weaker sector$^-$ phenotype, was not sensitive to any media conditions tested, and will be characterized at a later date.

The fourth mutant, *bms3-1*, was obtained from the screen for mutations causing increased sensitivity to loss of *BMH2*. This mutant was not completely lethal in the presence of *bmh2*-Δ, but it exhibited a greatly reduced rate of *BMH2* plasmid sectoring (Figure 1A). In a *BMH2* strain, *bms3-1* had a slight slow growth phenotype at all temperatures tested and was found to be highly sensitive to 3% formamide (data not shown). Formamide sensitivity was used to clone the *BMS3* gene, and it was found to be identical to *YPK1*, which encodes a serine/threonine protein kinase most related to mammalian serum and glucocorticoid inducible kinase (SGK) and Akt/PKB (Chen *et al.* 1993; Casamayor *et al.* 1999). Integrative transformation and segregation analysis confirmed that

A



B

	BMH1 YPK1	bmh1-Δ YPK1	BMH1 ypk1-Δ	bmh1-Δ ypk1-Δ
Live spores	27	21	15	0
Dead spores	0	0	6	27

FIGURE 1.—Synthetic growth defect of *ypk1* combined with 14-3-3 deletions. (A) Clockwise from top are SL2331 [*ypk1-2 bmh2*-Δ *ade2* + pDG46 (p*BMH2 ADE2*)]; SL2136 [*bmh2*-Δ *ade2* + pDG46 (p*BMH2 ADE2*)]; and SL2334 (SL2331 without plasmid). Cells were streaked onto YEPD and grown at 30° for 3 days to show the sectoring phenotypes. (B) A *bmh1*-Δ/*BMH1 ypk1*-Δ/*YPK1* strain (SL1388 × SL2545) was sporulated and tetrads were dissected. Numbers of viable and inviable spores of each genotype from 24 tetrads are indicated.

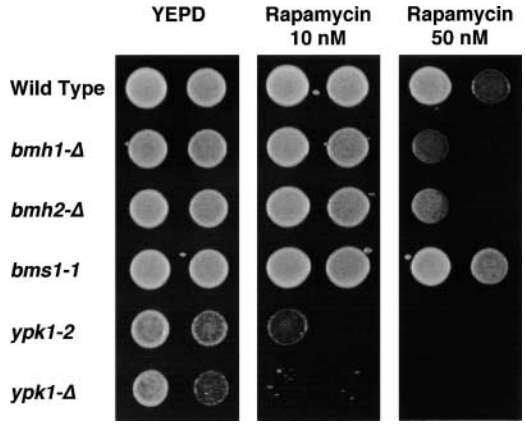


FIGURE 2.—*ypk1* but not *bms1* mutants are sensitive to rapamycin. Equal numbers of log-phase cells and a fivefold dilution of wild-type (SL1463), *bmh1*-Δ (SL1386), *bmh2*-Δ (SL1320), *bms1-1* (SL3246), *ypk1-2* (SL3365), and *ypk1*-Δ (SL2545) cultures were spotted onto YEPD containing 0, 10, or 50 nM rapamycin and grown at 25° for 2 days.

bms3-1 is allelic to *YPK1* (see MATERIALS AND METHODS); therefore, we hereafter refer to *bms3-1* as *ypk1-2*.

A null mutation of *YPK1* was generated in our genetic background, and the growth and formamide sensitivity phenotypes of the haploid mutants were identical to those of *ypk1-2*. Consistent with this, a cross of a *ypk1*-Δ mutant to a *bmh2*-Δ strain yielded slow-growing viable double-mutant spore progeny (not shown), similar to the leaky phenotype of *ypk1-2* (*bms3-1*) in the sectoring assay (Figure 1). A second Ypk-related kinase is encoded by *YPK2* (also referred to as *YKR2*; KUBO *et al.* 1989; CHEN *et al.* 1993). Similar to previous studies (CHEN *et al.* 1993; CASAMAYOR *et al.* 1999), we found that *ypk2*-Δ mutants grow well, but the double *ypk1*-Δ *ypk2*-Δ mutants are inviable (not shown). We also found that *ypk1-2 ypk2*-Δ double mutants are inviable. Thus, no differences between the *ypk1-2* and *ypk1*-Δ alleles were observed, suggesting that *ypk1-2* may be a complete loss-of-function allele.

Since *ypk1-2* was synthetically sick with *bmh2*-Δ we tested whether *ypk1* mutants are also synthetically sick or lethal with *bmh1*-Δ. SL1388 (*bmh1*-Δ) was crossed to SL2545 (*ypk1*-Δ) and subjected to tetrad analysis. We observed moderate levels of spore death in *ypk1*-Δ*::TRP1 BMH1* spores (16 viable Trp^+ spores from 21 expected) but complete lethality of *ypk1*-Δ *bmh1*-Δ spores (0 recovered from 24 expected) in 24 tetrads dissected (Figure 1B). Therefore, *ypk1*-Δ strains are sensitive to loss of either 14-3-3 gene and are more compromised in the absence of *BMH1*. This is likely due to the higher expression of Bmh1p relative to that of Bmh2p, which results in lower levels of 14-3-3 proteins in *bmh1*-Δ mutants than in *bmh2*-Δ mutants (GARRELS *et al.* 1994; GELPERIN *et al.* 1995).

As *ypk1-2* and *bms1-1* mutants are sensitive to reduction of 14-3-3 levels we asked if overexpression of 14-3-3 would suppress *ypk1-2* or *bms1-1* growth defects. Overexpression of either *BMH1* or *BMH2* did not affect the growth phenotypes of *ypk1*-Δ or *bms1-1* strains at various temperatures from 25° to 37° (not shown).

***ypk1* mutants are hypersensitive to rapamycin:** 14-3-3 proteins have been demonstrated to play a role in rapamycin-sensitive TOR pathway signaling in yeast (BERTRAM *et al.* 1998; BECK and HALL 1999; CHAN *et al.* 2000). Deletions of *BMH1* or *BMH2* cause hypersensitivity to rapamycin, while overexpression of *BMH1* or *BMH2* causes rapamycin resistance (BERTRAM *et al.* 1998; CHAN *et al.* 2000). Therefore, we examined whether *ypk1* or *bms1-1* mutants had altered sensitivity to rapamycin. We found that *ypk1-2* and *ypk1*-Δ mutants were hypersensitive to rapamycin, while *bms1-1* mutants were similar to wild-type strains (Figure 2). *bmh1*-Δ and *bmh2*-Δ strains had moderate sensitivity to rapamycin, as previously reported (BERTRAM *et al.* 1998; CHAN *et al.* 2000), but this was less severe than that seen for *ypk1* mutants (Figure 2). This suggested that Ypk1p may be involved in the rapamycin-sensitive TOR signaling pathway.

To explore the relationships among Ypk, 14-3-3 proteins, and the TOR pathway in more detail we first

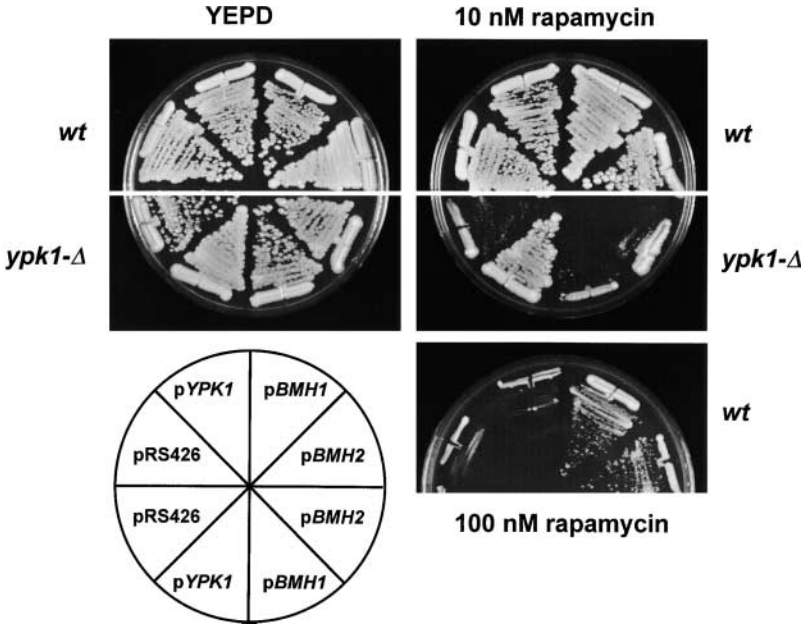


FIGURE 3.—Overexpression of *BMH1* or *BMH2* does not suppress the rapamycin sensitivity of *ypk1*-Δ and *YPK1* overexpression does not confer rapamycin resistance to wild-type cells. SL1462 (*wt*) or SL4235 (*ypk1*-Δ) were transformed with the indicated plasmids, streaked onto YEPD or YEPD containing various concentrations of rapamycin, and grown for 3 (YEPD) or 4 (rapamycin plates) days at 30°. Plasmids are: pRS426 (2μ, empty vector), p*YPK1* (pDG54/*YPK1*, 2μ), p*BMH1* (pDG59/*BMH1*, 2μ), and p*BMH2* (pJW18/*BMH2*, 2μ). Note that overexpression of *BMH* genes, but not *YPK1*, also suppressed the wild-type strain at 50 nM rapamycin.

examined whether *YPK1* overexpression could bypass rapamycin sensitivity of *bmh* mutants or wild-type cells and whether *BMH* overexpression could rescue *ypk1*-Δ. Overexpression of *BMH1* or *BMH2* could not suppress the rapamycin sensitivity of a *ypk1*-Δ strain, while the wild-type *YPK1* complemented the phenotype as expected (see Figure 3, 10 nM rapamycin). Increased dosage of the 14-3-3's was able to suppress the growth inhibition of a wild-type strain grown at higher concentrations of rapamycin (50 nM, not shown, or 100 nM rapamycin, Figure 3), consistent with previous studies (BERTRAM *et al.* 1998), but *YPK1*, 2μ could not bypass this rapamycin sensitivity (Figure 3). We also found that *YPK1* expressed from a 2μ plasmid could not rescue *bmh1*-Δ or *bmh2*-Δ rapamycin sensitivity (not shown).

Yeast *PKH1* and *PKH2* are a partially redundant essential gene pair encoding protein kinases related to mammalian PDK1, which is known to activate the Ypk-related kinases PKB/Akt and SGK as well as a number of other kinases (BELHAM *et al.* 1999; CASAMAYOR *et al.* 1999; INAGAKI *et al.* 1999; VANHAESEBROECK and ALESSI 2000). Recent studies have linked Pkh1p and Ypk1p to common signaling pathways (SUN *et al.* 2000; DEHART *et al.* 2002; SCHMELZLE *et al.* 2002) and Pkh1p can directly phosphorylate and activate Ypk1p *in vitro* (CASAMAYOR *et al.* 1999). These studies suggest that Pkh functions upstream of Ypk, although other work indicates that Ypk is not the only target of Pkh (INAGAKI *et al.* 1999). Therefore we examined whether an isogenic pair of *pkh1*-Δ and *pkh2*-Δ mutants are hypersensitive to rapamycin. We found that growth of the *pkh1*-Δ strain, but not *pkh2*-Δ, is inhibited at 20 nM rapamycin (Figure 4), suggesting Pkh1p also functions with Ypk in the pathway affected by rapamycin.

We also found that a strain carrying a deletion of the second *YPK* gene, *ypk2*-Δ, is not hypersensitive to rapamycin, as compared to an isogenic *ypk-ts* strain containing both a *ypk1-1*^{ts} allele and the *ypk2* deletion grown at a permissive growth temperature (Figure 4). These and the *pkh* results could indicate that the functions of the two Ypk or two Pkh proteins are not completely overlapping. More likely, Pkh1p and Ypk1p provide sufficient activity to confer rapamycin resistance even in the absence of their related counterparts, Pkh2p and Ypk2p, respectively. Consistent with this, the rapamycin sensitivity of the *ypk-ts* mutant is dependent upon its *ypk2*-Δ mutation (not shown).

Further tests showed that the overexpression of *TOR2* or a rapamycin-resistant allele of *TOR2* (*TOR2-1*^{r}) could suppress the rapamycin sensitivity of *ypk1*-Δ (Figure 5) or a *ypk-ts* mutant (not shown). This indicates that the *ypk* mutant strains are sensitive to rapamycin because of inhibition of TOR and not because of a nonspecific effect of rapamycin unrelated to TOR. However, *TOR* overexpression could not suppress the inviability of the *ypk-ts* strain at its nonpermissive growth temperature (not shown), suggesting that Ypk has essential functions independent of TOR.

We next tested whether *YPK1* overexpression could suppress the rapamycin sensitivity of a *tor1*-Δ strain. The *tor1*-Δ strain (note that *tor2*-Δ is inviable) was hypersensitive to rapamycin at concentrations as low as 10 nM rapamycin (Figures 4 and 5). Overexpression of *BMH* genes could partially suppress the rapamycin sensitivity of *tor1*-Δ (Figure 5), consistent with previous studies and the known role of Bmh proteins downstream of TOR (BERTRAM *et al.* 1998; BECK and HALL 1999). In contrast to 14-3-3, neither *YPK1* overexpression (Figure 5) nor *PKH1* overexpression (not shown) could suppress the *tor1*-Δ rapamycin hypersensitivity phenotype, suggesting

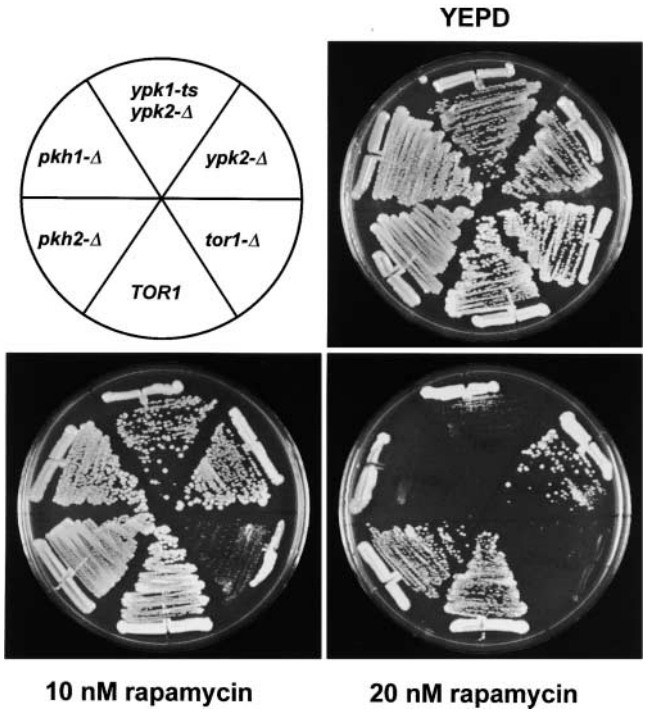


FIGURE 4.—*ypk1-ts ypk2*-Δ and *pkh1*-Δ but not *ypk2*-Δ or *pkh2*-Δ cells are hypersensitive to rapamycin. Strains *ypk1-1*^{ts} *ypk2*-Δ (YPT40) and *ypk2*-Δ (YES1), *tor1*-Δ (MH480) and *TOR1* (MH281), and *pkh1*-Δ (AC301) and *pkh2*-Δ (AC303) are isogenic pairs. Strains were streaked onto YEPD or YEPD with indicated concentrations of rapamycin and grown for 4 days at 25°.

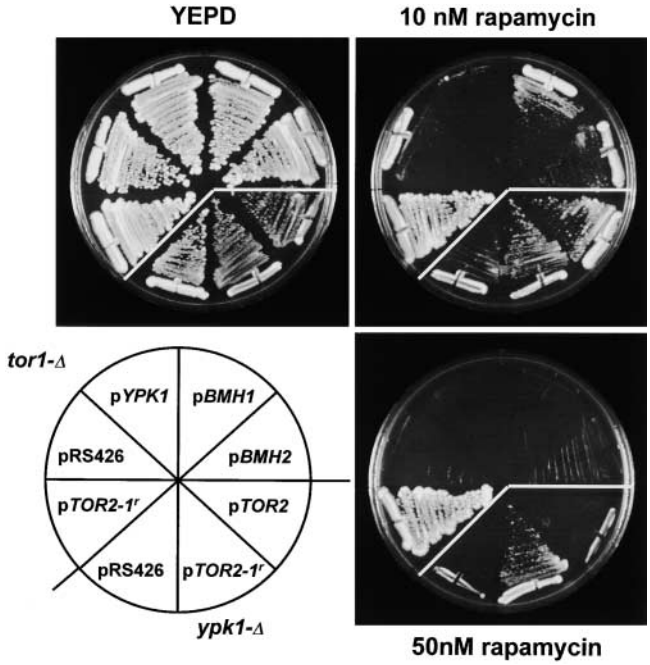


FIGURE 5.—Overexpression of *YPK1* does not suppress rapamycin hypersensitivity of *tor1*-Δ. *tor1*-Δ (MH480) or *ypk1*-Δ (SL4235) strains were transformed with the indicated plasmids, streaked onto YEPD or YEPD with different concentrations of rapamycin, and grown for 3 days at 30°. Plasmids are pRS426 (2μ, empty vector), p*YPK1* (pDG54/*YPK1*, 2μ), p*BMH1* (pDG59/*BMH1*, 2μ), p*BMH2* (pJW18/*BMH2*, 2μ), p*TOR2* (pJK3-3/*TOR2*, 2μ), and p*TOR2-1*^{r} (pJK12/p*TOR2-1* rapamycin-resistant allele, 2μ).

that they are not downstream of TOR. Supporting this, we found no difference in the kinase activity of Ypk1p isolated from cells treated with and without rapamycin (data not shown).

Ypkp-deficient cells are defective in initiation of translation: One of the major roles of the TOR signaling pathway is to regulate translation in response to nutrients (SCHMELZLE and HALL 2000; GINGRAS *et al.* 2001). Rapamycin treatment, starvation, or inactivation of both *TOR* genes lead to arrest of translation initiation (BARBET *et al.* 1996; DI COMO and ARNDT 1996). Therefore, we examined whether Ypk-deficient cells exhibit a translation initiation defect. Polysome analysis revealed a shift in the polyribosome peaks to those with only one to three ribosomes per mRNA in *ypk1*-Δ cells, compared with wild-type cells in which there was a significant pool of polyribosomes with ≥4–5 per translation complex (Figure 6A). This phenotype was more dramatic in the *ypk-ts* strain (*ypk1-1*^{ts} *ypk2*-Δ; Figure 6B). At 24° the polysome profile was normal. Upon shift of *ypk-ts* to 37° for 4 hr there was a nearly complete loss of polysomes and a dramatic increase in the 80 S monosome peak, while the isogenic *ypk2*-Δ control strain yielded normal polyribosome profiles after shift to 37° (Figure 6B).

A key regulator of 5′ cap-dependent mRNA translation initiation is eIF4G, which is a major component of the cap-binding complex and serves as an anchor for assembly of other initiation factors, including eIF4E and poly(A)-binding protein, onto mRNA (DEVER 1999; SACHS and VARANI 2000). Recent studies have shown that eIF4G protein is rapidly degraded upon treatment with rapamycin or during shift to diauxic growth, while eIF4E and eIF4A remain stable (BERSET *et al.* 1998; POWERS and WALTER 1999; KURUVILLA *et al.* 2001). This suggests eIF4G stability is regulated by TOR and perhaps other nutrient signaling pathways. We therefore tested whether loss of Ypk function affects the stability of eIF4G (Figure 7). By 2 hr after shift of the *ypk-ts* strain to 37° there was nearly a 10-fold decrease in eIF4G protein levels and by 4 hr the translation initiation factor had completely disappeared. In contrast, eIF4E showed only a slight gradual decline, such that by the 4-hr time point ~50% of initial eIF4E levels remained, possibly due to destabilization in the absence of its eIF4G scaffold protein. In the isogenic *ypk2*-Δ strain containing a normal *YPK1* gene, eIF4G and eIF4E were stable after shift to 37°. The rapid disappearance of eIF4G in the *ypk-ts* strain was not due to a general effect on translation or protein stability, as levels of Apm3p, a component of the AP-3 adaptor complex, or a ribosomal protein, Rpl3p, remained constant after shift to the nonpermissive tem-

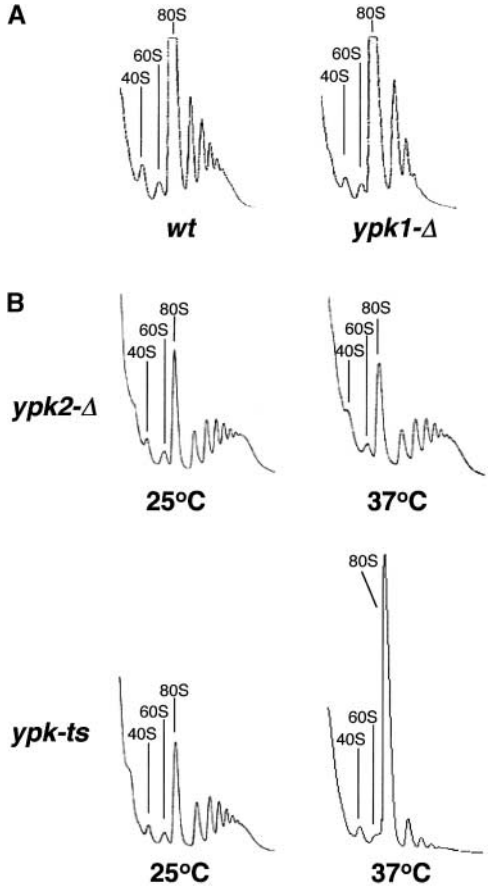


FIGURE 6.—Ypk-deficient yeast is defective in translation initiation. (A) Polysome profiles from congenic wt (SL1462) and *ypk1*-Δ (SL4235) strains grown at 30° in YEPD are shown. (B) Polysome profiles from isogenic *ypk2*-Δ (YES1) and *ypk-ts* (*ypk1-1*^{ts} *ypk2*-Δ, YPT40) strains grown at 25° or shifted to 37° for 4 hr are shown.

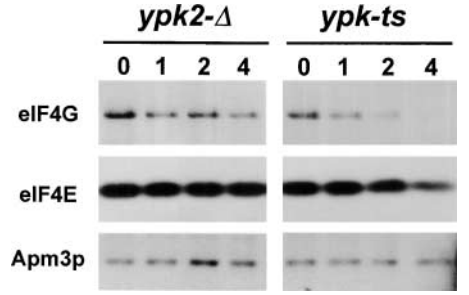


FIGURE 7.—eIF4G is depleted in *ypk-ts* cells at 37°. Isogenic *ypk2*-Δ (YES1) and *ypk-ts* (*ypk1-1*^{ts} *ypk2*-Δ, YPT40) strains were grown at 25° and shifted to 37°. Equal numbers of cells were removed at 0, 1, 2, and 4 hr after the shift and processed for immunoblotting with antibodies to eIF4G, eIF4E, and Apm3p as described in MATERIALS AND METHODS. Similar to Apm3p, the ribosomal protein, Rpl3p, was also stable throughout the time course (not shown).

perature (Figure 7 and not shown). These results indicate that loss of Ypk function leads to translation initiation arrest, at least in part due to depletion of eIF4G.

Although eIF4G declines upon rapamycin treatment or nutrient deprivation associated with diauxic growth (BERSET *et al.* 1998), it is not depleted by glucose starvation, which rapidly inhibits translation initiation (MARTINEZ-PASTOR and ESTRUCH 1996; ASHE *et al.* 2000). We therefore tested whether Ypk1p was affected by nutritional starvation. We found that nitrogen starvation caused a rapid depletion of Ypk1, while in the absence of glucose, Ypk1 levels were stable (Figure 8). eIF4G protein levels paralleled the Ypk1 results, with depletion during nitrogen starvation, but not during glucose starvation. eIF4E and the cytosolic control protein, Apm3p, were generally stable under both conditions. Since both nitrogen starvation and glucose deprivation inhibit protein synthesis, the selective loss of Ypk1 and eIF4G are not merely the result of inhibition of translation, but rather specific responses to the nitrogen status of the cell. This suggests that Ypk may be a component of a nutrient-sensing pathway that regulates translation through eIF4G.

DISCUSSION

To identify processes and pathways that are affected by 14-3-3, we performed synthetic lethal screens on the two 14-3-3 genes in *S. cerevisiae*, *BMH1* and *BMH2*. We anticipated we might identify mutants specific for either Bmh1p or Bmh2p or mutants that would be hypersensitive to 14-3-3 dosage. Since Bmh1p is expressed at levels three- to fourfold higher than those of Bmh2p (GARRELS *et al.* 1994; GELPERIN *et al.* 1995), we expected to find higher sensitivity to loss of *BMH1* than to loss of *BMH2*. This was found to be the case for two mutations examined further in this study. Classes of mutations that could be identified by this approach were hypothesized to affect proteins that require an interaction with 14-3-3 for proper regulation or function or kinases that phosphorylate target proteins to allow 14-3-3 binding. A third class includes proteins involved in a redundant or parallel pathway to one disrupted by reduction of 14-3-3. Finally, we expected to identify mutations in the other 14-3-3 gene, as complete loss of 14-3-3 protein is usually lethal (GELPERIN *et al.* 1995; VAN HEUSDEN *et al.* 1995; ROBERTS *et al.* 1997). As expected, we identified a mutation in a *BMH* gene, *bmh2*. In addition, the screens identified mutations in *BMS1*, *BMS2*, and *YPK1/BMS3* as being sensitive to reduced levels of 14-3-3.

Since 14-3-3 mutants are rapamycin hypersensitive and have been shown to be downstream effectors of TOR signaling, we tested our *bms* mutants for sensitivity to this drug. We found that *ypk1-2/bms3-1* causes rapamycin hypersensitivity. One of the major effects of rapamycin, by its effect on TOR, is to inhibit translation initiation. This led us to discover that *ypk1*-Δ also causes a translation initiation defect, and these phenotypes were even more pronounced in a *ypk-ts* strain at its

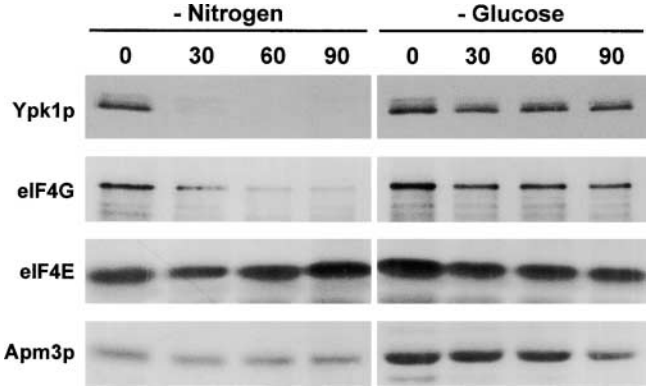


FIGURE 8.—Ypk1p is depleted upon shift to nitrogen starvation conditions, but not during glucose starvation. A log-phase culture of SL1462 transformed with pAD1 (p*YPK1-HA*) was grown at 30° in C-HIS and then shifted to nitrogen starvation medium or glucose starvation medium. Cells were harvested at times indicated and extracts were prepared for immunoblot analysis as described in MATERIALS AND METHODS. Blots were probed with antibodies to HA to detect Ypk1-HA, anti-eIF4G, anti-eIF4E, and anti-Apm3p. All proteins were stable if maintained on normal C-HIS medium (not shown).

nonpermissive temperature. In contrast, *bms1-1* was not rapamycin sensitive, even though we have previously shown that *bms1-1* also causes a translation defect by its effect on an early step in 40S ribosomal subunit biogenesis (GELPERIN *et al.* 2001; also see WEGIERSKI *et al.* 2001). Furthermore, although *ypk1-2* was hypersensitive to rapamycin, it was not hypersensitive to other translational inhibitors, such as paromomycin or neomycin, which did affect *bms1-1* (not shown). Thus the rapamycin hypersensitivity of the *ypk* mutants appears to be a specific phenotype and is not merely due to the combined effects of rapamycin and *ypk* mutations on translation or to a general drug sensitivity.

Ypk1/2p have highest homology within their catalytic domain to mammalian SGK and Akt/PKB protein kinases (55 and 52%, respectively) and can be functionally replaced by SGK and partially by Akt/PKB (CASAMAYOR *et al.* 1999). In mammalian cells the 3-phosphoinositide-dependent kinase PDK1 phosphorylates and activates both SGK and Akt/PKB, among other kinases, in response to growth factors and survival factors (BELHAM *et al.* 1999; VANHAESEBROECK and ALESSI 2000). In yeast a functional homolog of PDK1, Pkh1p, activates Ypk1p *in vitro* (CASAMAYOR *et al.* 1999), and both Ypk1p and Pkh1p appear to be downstream effectors of a lipid signaling pathway involving sphingolipids (SUN *et al.* 2000). Akt/PKB has multiple downstream targets, possibly including mammalian mTOR/FRAP (SCOTT *et al.* 1998; KANDEL and HAY 1999; NAVE *et al.* 1999; SEKULIC *et al.* 2000), although the importance of the Akt-dependent phosphorylation of TOR is somewhat controversial (see GINGRAS *et al.* 2001 and references therein). Nevertheless, the rapamycin sensitivity of *ypk* and *pkh1* strains we have observed further suggests that Pkh1p and Ypkp function in a pathway analogous to PDK1 and Akt/PKB.

Interestingly, Akt/PKB is responsible for phosphorylating 14-3-3 target proteins on residues that allow 14-3-3 to bind (ZHA *et al.* 1996; DATTA *et al.* 1997; BRUNET *et al.* 1999). CASAMAYOR *et al.* (1999) have done preliminary characterization of the sequence that is recognized and phosphorylated by Ypk1p and found it to be RXRXX[S/T][aromatic]. This is reminiscent of a 14-3-3-binding motif if it is followed by a proline at the +2 position after the phosphoserine or phosphothreonine (YAFFE *et al.* 1997). Thus, Ypk1p, like Akt/PkB, may phosphorylate and regulate the binding of 14-3-3 to an as yet unidentified target or targets, which could explain the synthetic phenotypes observed when *ypk1* was combined with *bmh* deficiency.

Alternatively, the genetic interaction we observed between *ypk1* and 14-3-3 mutations could relate to the fact that both gene products have functions that intersect with components regulated by TOR. Both *YPK* and 14-3-3 mutants are hypersensitive to rapamycin. 14-3-3 is a downstream component of the TOR pathway and acts to sequester the Msn2/4p stress-responsive transcription factors in the cytosol in response to TOR activation (BECK and HALL 1999). Loss of Ypk function causes a dramatic decrease in initiation of translation and a concomitant depletion of eIF4G, similar to that observed after treatment of cells with rapamycin (BARBET *et al.* 1996; BERSET *et al.* 1998; POWERS and WALTER 1999; KURUVILLA *et al.* 2001). Interestingly, we found that Ypk1 protein levels are regulated by nitrogen availability, but they are not affected by glucose depletion, similar to the pattern seen for eIF4G. This suggests that Ypk is a component of a nutrient-sensing pathway that may regulate translation initiation by affecting eIF4G levels in the cell. The fact that Ypk and TOR affect a common downstream target (eIF4G) further supports a role for Ypk in a pathway that intersects with TOR signaling.

A key question that remains is whether Ypk directly affects the TOR pathway itself, or whether it is a component of a functionally related pathway that operates parallel to TOR. We found that overexpression of *YPK1* was unable to suppress the rapamycin sensitivity of a wild-type strain or of a *tor1*-Δ strain. *PKH* overexpression gave similar results (not shown). Moreover, we found no effect of rapamycin on the levels or activity of Ypk1 (not shown). These data seem to indicate that Ypk and Pkh do not function downstream of TOR.

Other evidence points to a model in which Ypk and Pkh function in a pathway parallel to TOR. Overexpression of 14-3-3 proteins could not suppress the rapamycin sensitivity of *ypk* mutants to any extent, as compared to their ability, as downstream effectors of TOR, to confer rapamycin resistance to *tor1*-Δ and wild-type strains (BERTRAM *et al.* 1998; BECK and HALL 1999). Moreover, overexpression of TOR could not suppress the growth

defect or the eIF4G-induced depletion in the *ypk-ts* strain at 37° (not shown). In preliminary studies, we find identical results for a *pkh-ts* strain, which is also rapamycin hypersensitive at permissive growth temperatures and causes depletion of eIF4G at the nonpermissive temperature (not shown). Together, these results suggest that Ypk's regulation of translation initiation may not be directly through TOR. If Ypk is on a parallel pathway, independent inputs from both Ypk and TOR might be required for activation of a common downstream target, as has been suggested for the phosphoregulation of S6 kinase, 4E-BP1, and eIF4G by the related mammalian signaling pathways involving PI3 kinase, Akt(PKB), and mTOR (see Gingras *et al.* 2001 and references therein).

Recent studies indicate that Ypk and Pkh have other cellular functions. The sphingolipid signaling pathway that activates Ypk has recently been shown to be required for endocytosis and normal actin organization, and both Ypk function and Pkh function have been implicated in this pathway as well (Inagaki *et al.* 1999; Friant *et al.* 2000, 2001; Zanolari *et al.* 2000; deHart *et al.* 2002). Furthermore, both kinases appear to be upstream activators of the PKC signaling pathway (Inagaki *et al.* 1999; Schmelzle *et al.* 2002). However, *pkc1-ts* mutants are suppressed by sorbitol addition, but neither the endocytic nor the eIF4G depletion phenotypes of *ypk-ts* are suppressed by sorbitol (not shown; deHart *et al.* 2002). Also, *pkc1-ts* mutants are not rapamycin sensitive (not shown) and have normal endocytosis at the nonpermissive temperature (Friant *et al.* 2000). This suggests that Pkh and Ypk are likely to receive inputs from multiple signals and regulate multiple downstream targets, similar to PDK1 and Akt/PKB and other kinase signaling pathways in yeast and animals. Future work will be aimed at identifying the upstream activators and downstream effectors of the Ypk pathway leading to regulation of translation initiation, as well as whether any targets of Ypk phosphorylation are subject to regulation by 14-3-3 binding.

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