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. 1995), 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 Bnh1p 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 Hermoort 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 target of rapamycin (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; Hellwell et al. 1994). Loss of TOR function resembles the phenotype of cells treated with rapamycin, although TOR2 also has a second rapamycin-
TABLE 1

Yeast strains used in this study

<table>
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<th>Genotype</th>
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<td>Helliwell et al. (1994)</td>
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<td>MH580</td>
<td>MATα leu2-3,112 ura3-52 rmel trp1 his4 HMLa</td>
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<td>YES1</td>
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<td>pDG46</td>
<td>This study</td>
</tr>
</tbody>
</table>

* 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 (YPD) and synthetic selective dropout media were prepared as described in Nelson and Lemmon (1993). YEPD + 5% 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, TRPI) was created by cloning a 4.2-kb Kpn1-ClaI 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 pRS316 (Sikorski and Hieter 1989). Second, a 4.2-kb Kpn1-ClaI fragment containing BMH2 was ligated into the Kpn1 and ClaI sites. pDG53 (LEU2, YPK1) was generated by cloning a 3.7-kb BglII-XhoI YPK1 fragment into the BamHI-XhoI sites of the LEU2 integrating vector, pRS305 (Sikorski and Hieter 1989). pDG54 (YPKI, 2µ, URA3) contains the 3.7-kb BglII-XhoI YPK1 fragment cloned into the BamHI-XhoI sites of pRS426 (Christianson et al. 1992). pDG56 (CEN, ADE3, TRIP1, BMH1) was created by cloning a 5.5-kb BamHI-SalI fragment containing ADE3 into pWJ42 (described in Gelperin et al. 1995). pDG58 (BMH2, CEN, URA3) was created by cloning a 3721-bp SalI-XhoI fragment containing BMH2 into pRS316. pDG59 (BMH1, 2µ, URA3) contains a 3.2-kb...
TABLE 2
Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Markers</th>
<th>Source</th>
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<td>pAD1</td>
<td>2µ YPK1-3HA:His3MX URA3</td>
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<td>pDG45</td>
<td>CEN TRP1 BMH2</td>
<td>This study</td>
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<td>pDG46</td>
<td>CEN ADE2 URA3 BMH2</td>
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<td>2µ URA3 YPK1</td>
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<tr>
<td>pW18</td>
<td>2µ URA3 BMH2</td>
<td>GELPERIN et al. (1995)</td>
</tr>
<tr>
<td>pL72</td>
<td>ypk1-D::TRP1</td>
<td>CHEN et al. (1993)</td>
</tr>
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</table>

EcoRI-KpnI fragment of BMH1 cloned into pRS426, pDG60 (BMH1, CEN, URA3) was created by cloning a 3205-bp EcoRI-KpnI 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'-GGAACCTT TACTACAGTTAGTAGCTCAATGGTGCAAGGTAGAAGC-3' and 5'-CGAACAT ATTAGACGGATCCCCGGGTTAATTAA-3' using pFA6a-3HA-His3MX6 (LONETINE et al. 1998) as a template.

Synthetic lethal screens: Two synthetic lethal screens were carried out. The first was performed with bmh1-D using the ade2 ade3 ade2 ade3 red/white colony sectoring method (BENDER and PRINGLE 1991). A bmh1-D 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 formamide sensitivity and the 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, bmh3-1 (ypk1-2, see below), was analyzed further. This candidate was backcrossed three times to the parental strain SL2136 before use in further studies.

Cloning bmh2 synthetic mutations: Cloning of wild-type gene for bmh1-I, which causes temperature-sensitive growth, is described elsewhere (GELPERIN et al. 2001). The bmh3-1 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 BM3 gene, the bmh3-1 strain, SL2381, was transformed with a YIp13-based genomic library (MATSUURA and ANKRU 1993) and transformants were selected directly onto CLEU + 5% formamide plates. One library plasmid was able to rescue both the formamide sensitivity and the sector− phenotype of SL2381. 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 restesting sectoring and formamide sensitivity in SL2381. The complementing open reading frame was found to be YPK1.

YPK1 gene deletion and confirmation that bmh3-1 is allelic to YPK1: YPK1 was deleted in SL1528 using pL72 (CHEN et al. 1993) digested with Pvu2 to generate a ypk1-D::TRP1 deletion fragment. Correct integration was confirmed by Southern blotting and haploid segregants containing the ypk1-D::TRP1 allele were generated by tetrad analysis.

To confirm that the bmh3-1 mutation is allelic to YPK1, pDG53 (Yip-YPK1) was linearized within YPK1 with NsiI and transformed into SL2351 (bmh2-D::HIS3 bmh3-1 + pDG56 (BMH2)). Proper integration was confirmed by Southern blot. Resulting integrants were mated to a bmh3-1 BMH3 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°C 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 pelleted. 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 A260 units were loaded onto a 16.2-ml 10–50% sucrose gradient containing 100 mM KCl, 5 mM MgCl2, 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 ypk2Δ (YES1) strains, cells were grown to midlog phase in YEPD at 25°C and a zero time sample was harvested. Then cells were washed and inoculated into fresh YEPD prewarmed to 37°C at 0.25 × 107 cells/ml at each time point before and after the shift to 37°C. 1 × 106 cells were harvested and washed in dH2O and the final cell pellet was frozen in a microcentrifuge tube at −80°C. 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 (Stepf 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°C 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 (YPKI-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 dH2O and resuspended at 0.25 × 107 cells/ml in normal growth medium (C-HIS, not shown), synthetic yeast nitrogen base medium lacking ammonium sulfate and amino acids plus 2% glucose (nitrogen starvation medium), or C-HIS minus glucose (glucose starvation). At each time point 1 × 106 cells were harvested and washed one time with dH2O 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 MgCl2, 150 mM NaCl, protease inhibitors (see above), and phosphatase inhibitors (50 mM NaF, 1 mM NaVO4). 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°C 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 Panek et al., 1997); mouse anti-Rpl3p 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 BMW1 and/or BMW2 (see MATERIALS AND METHODS for details). One mutation from the screen for bmh1Δ synthetic lethal mutants failed to complement a bmh2Δ mutation, implying that the mutation was in BMW2. A bmh2 mutation was expected from this screen, since bmh1Δ bmh2Δ 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°C–35°C (not shown). This mutation is not completely lethal in the presence of bmh1Δ or bmh2Δ, but the combined mutations lead to synergistic growth defects at the bmh1Δ semipermissive temperatures of 30°C and 32°C, with the effect of bmh1Δ being more severe than that of bmh2Δ (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 BMW2. This mutant was not completely lethal in the presence of bmh2Δ, 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 YPKI, 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
ypk1-2 bmh2-Δ + pBMH2
bmh2-Δ + pBMH2

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 (pBMH2 ADE2)]; SL2136 [bmh2-Δ ade2 + pDG46 (pBMH2 ADE2)]; and SL2334 (SL2331 without plasmid). Cells were streaked onto YEPD and grown at 30°C 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.

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 (GARRELLS 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°C to 37°C (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...
examined whether \( YPK1 \) overexpression could bypass rapamycin sensitivity of \( bmh \) mutants or wild-type cells and whether \( BMH \) overexpression could rescue \( ypk1-\Delta \). Overexpression of \( BMH1 \) or \( BMH2 \) could not suppress the rapamycin sensitivity of a \( ypk1-\Delta \) 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 \( YPK1 \), 2\( \mu \) could not bypass this rapamycin sensitivity (Figure 3). We also found that \( YPK1 \) expressed from a 2\( \mu \) plasmid could not rescue \( bmh1-\Delta \) or \( bmh2-\Delta \) 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 \textit{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 \( pkh1-\Delta \) and \( pkh2-\Delta \) mutants are hypersensitive to rapamycin. We found that growth of the \( pkh1-\Delta \) strain, but not \( pkh2-\Delta \), 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, \( ypk2-\Delta \), is not hypersensitive to rapamycin, as compared to an isogenic \( ypk-ts \) strain containing both a \( ypk1-1^w \) allele and the \( ypk2-ts \) 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 \( ypk2-\Delta \) mutation (not shown).

Further tests showed that the overexpression of \( TOR2 \) or a rapamycin-resistant allele of \( TOR2 \) (\( TOR2-r^\) ) could suppress the rapamycin sensitivity of \( ypk1-\Delta \) (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 \( tor1-\Delta \) strain. The \( tor1-\Delta \) strain (note that \( tor2-\Delta \) 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 \( tor1-\Delta \) (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 \( tor1-\Delta \) rapamycin hypersensitivity phenotype, suggesting...
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).

Ypk1p-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 ypk1Δ 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 (ypk1Δ ypk2Δ; Figure 6B). At 24°C the polysome profile was normal. Upon shift of ypk-ts to 37°C for 4 hr there was a nearly complete loss of polysomes and a dramatic increase in the 80 S monosome peak, while the isogenic ypk2Δ control strain yielded normal polyribosome profiles after shift to 37°C (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°C 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 ypk2Δ strain containing a normal YPK1 gene, eIF4G and eIF4E were stable after shift to 37°C. 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 temperatur...
is depleted in ypks cells at 37°C. Isogenic ypks (YES1) and ypks (ypk1-1ts ypks, YPT40) strains were grown at 25°C and shifted to 37°C. Equal numbers of cells were removed at 0, 1, 2, and 4 hr after the shift and processed for immunoblotting with antibodies to elf4G, elf4E, and Apm3p as described in materials and methods. Similar to Apm3p, the ribosomal protein, Rpl3p, was also stable throughout the time course (not shown).

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 Bnh1p 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, bnh2. 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 (pYPK1-HA) was grown at 30°C 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-elf4G, anti-elf4E, 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, Phk1p, activates Ypk1p in vitro (Casamayor et al. 1999), and both Ypk1p and Phk1p 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 phk1 strains we have observed further suggests that Phk1p 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 bmb 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 elf4G, 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 elf4G. This suggests that Ypk is a component of a nutrient-sensing pathway that may regulate translation initiation by affecting elf4G levels in the cell. The fact that Ypk and TOR affect a common downstream target (elf4G) 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 tor1Δ 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 Phk do not function downstream of TOR.

Other evidence points to a model in which Ypk and Phk 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 tor1Δ 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°C (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; Schmelze et al. 2002). However, pkel-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, pkel-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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