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Coordinate Regulation of Sugar Flux and Translation by PAS Kinase

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Summary

PAS kinase is a serine/threonine kinase regulated in *cis* by a PAS domain. A genetic study of the two PAS kinase genes in budding yeast gave evidence of the involvement of these enzymes in the control of sugar metabolism and translation. Using a biochemical screen for PAS kinase substrates, three translation factors were identified as direct phosphorylation targets. PAS kinase was also found to phosphorylate UDP-glucose pyrophosphorylase and glycogen synthase, the enzymes catalyzing the two final steps in the glycogen biosynthetic pathway. Genetic, biochemical, and physiological data provide evidence that both of these enzymes are inhibited by PAS kinase-dependent phosphorylation, thereby downregulating carbohydrate storage. These studies provide evidence of a cell-autonomous signaling system that both controls and connects the balance of fuel consumption/storage to protein synthesis.

Introduction

Living cells constantly monitor internal and external environments and adapt with regulatory decisions to optimize opportunity for viability and appropriate physiological function. For cells incapable of photosynthesis, critical attention is paid to the availability of reduced fuels, such as carbohydrates. When sugars are brought into a cell, their energy can be burned via glycolysis for the generation of ATP. Alternatively, sugar and its energy can be deposited in storage forms, such as glycogen. In addition to the consumption/storage decision cells must make when challenged with different amounts or sources of carbohydrate, they must also modulate their level of protein synthesis. Translation consumes 5% of the human caloric intake (Mathews et al., 2000) and as much as half of the energy utilized by rapidly growing bacterial cells (Meisenberg and Simmons, 1998). Log-growth yeast cells contain nearly 200,000 ribosomes, occupying between 30 and 40% of total cytoplasmic volume (Warner, 1999). The simple production of the translational apparatus absorbs 60% of total transcriptional activity in actively growing eukaryotic cells. It is clear that so costly a metabolic process must be closely monitored and regulated as a function of nutritional, hormonal, and developmental states.

The availability of nutrients weighs heavily on the regulatory decisions made by living cells. Not surprisingly, an energetically expensive process such as protein synthesis is largely undertaken when nutrients are abundant. At least three translational regulatory targets, eIF4F, eIF2/eIF2B, and $p70^{S6k}$, are controlled by amino acid supply (Kimball and Jefferson, 2000). Carbohydrate availability also exerts rapid and stringent control of protein synthesis (Ashe et al., 2000). Similarly, nutrient limitation can lead to an increase in the deposition of storage carbohydrates at the expense of glycolytic flux (Francois and Parrou, 2001; Lillie and Pringle, 1980).

For the yeast *S. cerevisiae*, nutrient sensing and decision making is cell-autonomous. For complex multicellular organisms, decisions regarding the fates of reduced fuel and protein synthesis must be responsive to both the metabolic environment of the individual cell as well as the physiological status of the organism as a whole. The levels of circulating insulin, for example, directly correlate with nutrient availability and serve to coordinate physiological output throughout the body. The regulatory effects of insulin are, however, dependent upon cell-autonomous sensory systems wherein nutritional status is monitored at the level of individual cells. As an example, insulin-mediated regulation of the eIF2 and eIF4F translation factors is gated by the availability of amino acids and glucose to the responsive cell (Patel et al., 2001; Wang et al., 1998).

The pathways that mediate cell-autonomous energy sensing in eukaryotic cells include the SNF1/AMPK family of protein kinases and the mTOR protein kinase. In response to sufficient nutrients, mTOR stimulates the production of ribosomes and activates protein synthesis (Dennis et al., 1999). AMPK is activated by 5′-AMP, the levels of which inversely correlate with ATP content and initiates a cellular program to elevate ATP levels (Hardie et al., 1998). A number of energy-sensing systems have also been discovered in prokaryotic cells. Many of these prokaryotic systems utilize a PAS domain as the molecular sensor of oxygen, redox status, ATP, or other indicators of cellular metabolic status (Stephenson and Hoch, 2001; Taylor and Zhulin, 1999). Upon appropriate stimulation, PAS domains exert allosteric regulation in *cis* on a histidine kinase that, in turn, initiates a phospho-relay cascade resulting in an appropriate, adaptive response (David et al., 1988).

PAS kinase is the single metazoan protein known to contain both protein kinase and PAS domains (Rutter et al., 2001). Here, we report genetic and biochemical studies of the yeast orthologs of PAS kinase. Evidence is presented favoring the identification of PAS kinase as a global regulator of both protein synthesis and sugar flux. When active, this enzyme coordinately instructs yeast cells to burn reduced fuels and enhance protein synthesis.

Results

Phenotypic Analysis of PSK Mutant Yeast

We generated strains of the yeast *S. cerevisiae* wherein one or both of the PAS kinase genes (PSK1 and PSK2)

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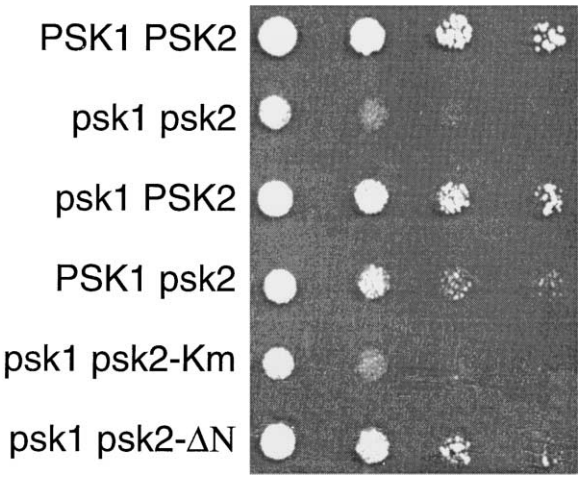

Figure 1. PSK Kinase Activity Is Necessary for Growth under gal^{ts}-Restrictive Conditions

Strains of the indicated genotype were grown to saturation in minimal 2% glucose medium lacking uracil and diluted in water. These diluted samples were spotted at 10,000, 2000, 400, and 80 cells per spot onto minimal medium plates lacking uracil with 2% galactose as carbon source. The plate was incubated for 6 days at 39°C. The strains shown are from top to bottom: parental strain (BHY10 + pRS416), double mutant (JRY40 + pRS416), psk2 single mutant (JRY10 + pRS416), psk1 single mutant (JRY30 + pRS416), double mutant with a plasmid containing a PSK2 allele bearing a kinase-inactivating mutation (JRY40 + pRS416-PSK2 K870R), double mutant with a plasmid containing a PSK2 deletion expressing only the kinase domain (JRY40 + pRS416-PSK2 ΔN792). JRY10 + pRS416 and JRY30 + pRS416 are phenotypically indistinguishable from JRY40 + pRS416-PSK1 and JRY40 + pRS416-PSK2, respectively.

were eliminated by homologous recombination. The single (psk1 PSK2 or PSK1 psk2) and double knockout (psk1 psk2) strains were indistinguishable from their parental counterparts when maintained under standard laboratory growth conditions. The three strains were compared with the parental strain under roughly one hundred compromised growth conditions as described by Hampsey (1997). We also assayed the four strains on all medium conditions at both high (39°C) and low (20°C) stress-inducing temperatures.

One condition emerged from this synthetic phenotype screen as selectively detrimental to growth of the psk1 psk2 double-mutant strain. Glucose is the preferred carbon source of *S. cerevisiae* and is utilized exclusively when available (Johnston and Carlson, 1992). Among the many alternative carbon sources used to challenge the three mutants, only galactose failed to support growth of the psk1 psk2 double mutant and, to a lesser extent, of the PSK1 psk2 single mutant. These defects were exacerbated at elevated temperature (39°C) and on minimal medium (Figure 1). The temperature-sensitive galactose utilization (gal^{ts}) phenotype of the psk1 psk2 double mutant was efficiently rescued upon provision of the PSK2 gene expressed from an extrachromosomal, centromere-bearing plasmid. Enzymatic activity of the PAS kinase polypeptide (Psk2p) was required for functional rescue; in contrast, the PAS domains of Psk2p were not (Figure 1).

Identification of High-Copy Suppressors

The identification of the psk1 psk2 mutant as a temperature-sensitive galactose utilization-defective variant of *S. cerevisiae* provided a means by which to search for genes that might be functionally related. Genes were identified which, when provided on a high-copy plasmid, would complement the gal^{ts} growth defect of the psk1 psk2 mutant strain. Individual suppressing plasmids were isolated, retested, and identified by DNA sequencing. A list of confirmed high-copy suppressors is shown in Table 1.

As expected, we recovered the PSK1 and PSK2 genes as gal^{ts} suppressors. We also recovered the SIP1 gene, which encodes one of three β-subunits of the SNF1 kinase complex. SNF1 has many functions including enabling growth on suboptimal carbon sources including galactose (reviewed in Hardie et al., 1998). The β-subunits activate and target SNF1 kinase to appropriate substrates (Schmidt and McCartney, 2000). As such, SIP1 overexpression probably increases the efficiency of galactose utilization in a manner not directly related to the psk1 psk2 mutant. Indeed, the growth rate of wild-type yeast on minimal galactose medium at 39°C was slightly enhanced by SIP1 overexpression.

We also recovered, as high-copy suppressors, the genes encoding both isoforms of phosphoglucomutase, PGM1 and PGM2. This enzyme catalyzes the interconversion of glucose-6-phosphate (glucose-6-P) and glucose-1-phosphate (glucose-1-P; Figure 8). As such, phosphoglucomutase is necessary for the utilization of galactose (Boles et al., 1994; Masuda et al., 2001). Increased expression of this enzyme in the psk1 psk2 double mutant might suppress a galactose utilization phenotype by increasing flux of galactose to glucose-6-P (see Discussion).

The majority of the remaining high-copy suppressors encode gene products involved directly or indirectly in protein synthesis, including both RNA and polypeptide components of the translation apparatus (Table 1). The gene that exerted the strongest suppressing effect, restoring growth of the psk1 psk2 double mutant to wild-type levels, was also recovered most frequently in the high-copy suppressor screen. The ORF present in sixteen independently isolated plasmids is designated YDL189w in the *S. cerevisiae* standardized gene nomenclature system. Due to its suppressive activity, we designated this gene as *R*NA-*B*inding *S*uppressor of PAS kinase (RBS1). Whereas nothing has been published regarding the function of RBS1, analysis of its primary amino acid sequence revealed the presence of an R3H domain near the N terminus. The R3H domain is an evolutionarily conserved, single-stranded nucleic acid binding domain (Grishin, 1998). Hypothesizing that this nucleic acid binding domain might be required for RBS1 function, we generated a mutant RBS1 allele wherein the conserved R57 and H61 residues, located within the R3H domain, were changed to alanine. This mutant is predicted to adopt a native-like conformation, but should be substantially compromised in its ability to bind nucleic acid. The RBS1 R57A/H61A mutant allele was completely unable to support growth of a psk1 psk2 strain under the gal^{ts}-restrictive condition.

The suppression of the psk1 psk2 mutant gal^{ts} phenotype by high-copy expression of any of a number of positive translation factors raised the possibility that PAS kinase might function to enhance protein synthesis. If so, high-copy expression of PAS kinase itself might suppress deficits caused by the inactivation of proteins

Table 1. Genetic Suppressors of the psk Mutant Growth Phenotype

Gene	#	Description	Efficacy
PSK1/PSK2	5(2/3)	PAS kinase	
PGM1	1	Phosphoglucomutase	W
PGM2	2	Phosphoglucomutase	W
SIP1 (ΔN)	8	Glucose deprepression	S[a]
DDP1	4	Diadenosine polyphosphate hydrolase	W
ADE16	2	Adenine and histidine biosynthesis	W
CPA1	5	Arginine biosynthesis-translationally regulated	M
RBS1	16	R3H domain protein	S
DED1	2	RNA helicase-translation initiation	M
DBP1	4	RNA helicase-translation initiation	M
EDC1	3	Enhancer of mRNA decapping	M
POP4	4	Rnase P/Rnase MRP protein subunit	S
RPR1	2	Rnase P/RNA subunit	M
UBA2 (ΔN)	2	Sumoylation/mRNA polyadenylation	S
UBA2 (FL)	-	Sumoylation/mRNA polyadenylation	-
REF2	2	mRNA polyadenylation	W
RDN58	9	Ribosomal DNA (5.8S rRNA subunit)	W

Table includes all genes recovered as at least two independent isolates. The column labeled # specifies the number of independent suppressing clones that were recovered for each gene. Efficacy is a qualitative visual assessment of the ability of each gene to complement the psk1 psk2 gal^{ts} phenotype. S = strong (growth similar to a PSK1 PSK2 strain); M = medium; W = weak.
[a] SIP1 enhanced growth of a wildtype PSK1 PSK2 strain on minimal galactose-containing medium at 39°C. See Supplemental Data available at http://www.cell.com/cgi/content/full/111/1/17/DC1 for discussion of SIP1 and UBA2 isolation.

known to be important for efficient translation. An *S. cerevisiae* strain lacking the STM1 (TIF3) gene, which encodes the yeast eIF4B translation initiation factor, exhibits a temperature-sensitive (ts) growth phenotype (Altmann et al., 1995). Provision of PSK2 on a 2 μ origin-bearing plasmid substantially complemented the ts growth defect of the stm1 mutant strain (Figure 2A). This growth deficit was previously shown to be accompanied by an attenuation of protein synthesis (Altmann et al., 1993; Coppolecchia et al., 1993), which was confirmed by SDS-PAGE analysis of [^{35}S]-methionine and cysteine incorporation (Figure 2B, compare lanes 1 and 3). As shown in lanes 3 and 4 of Figure 2B, it was observed that this deficit in [^{35}S] incorporation was largely overcome by overexpression of PSK2.

Identification of PAS Kinase Substrates

Complementing the identification of high-copy suppressors of the psk1 psk2 double mutant, we undertook biochemical experiments to discover putative phosphorylation targets of the enzyme. Our simple strategy entailed the generation of a soluble lysate from *S. cerevisiae*, and subsequent fractionation using various chromatographic separation techniques to generate partially purified pools of proteins that maintained native multiprotein interactions. We then tested each fraction for Psk2p-dependent protein phosphorylation (see Experimental Procedures). We found four distinct polypeptides phosphorylated only in the presence of Psk2p.

One fraction, which contained proteins retained on Ni-NTA agarose, is shown in Figure 3. Although a number of the proteins in the sample were phosphorylated in a Psk2p-independent manner (lane 2), one polypeptide, migrating with an apparent molecular weight of 55 kDa, was phosphorylated only in the sample supplemented with the Psk2p enzyme (lane 3). We identified this protein as the product of the UGP1 gene, *U*DP-Glucose *P*yrophosphorylase (Daran et al., 1995). Ugp1p catalyzes the

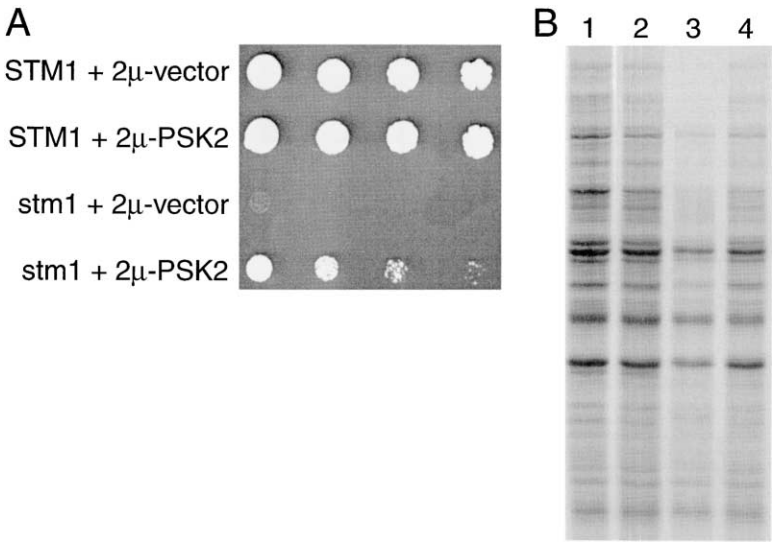

Figure 2. PSK2 Suppresses Growth and Protein Synthesis Deficits of eIF4B Mutant

(A) Strains of the indicated genotype were grown to saturation in minimal 2% glucose medium lacking tryptophan and diluted in water. Diluted samples were spotted at 10,000, 2000, 400, and 80 cells per spot onto minimal medium plates lacking tryptophan with 2% glucose and incubated for 4 days at 39°C. The strains shown are from top to bottom: CW04 + pRS424, CW04 + pRS424-PSK2, RCB1-1c + pRS424, and RCB1-1c + pRS424-PSK2.

(B) The strains from (A) were grown to log phase in minimal 2% glucose medium lacking tryptophan and methionine. 0.5 OD were transferred to 37°C for 15 min followed by addition of [^{35}S]-methionine and -cysteine. Labeling continued for 15 min at 37°C followed by SDS-PAGE and autoradiography. Lanes are: (1) CW04 + pRS424, (2) CW04 + pRS424-PSK2, (3) RCB1-1c + pRS424, and (4) RCB1-1c + pRS424-PSK2.

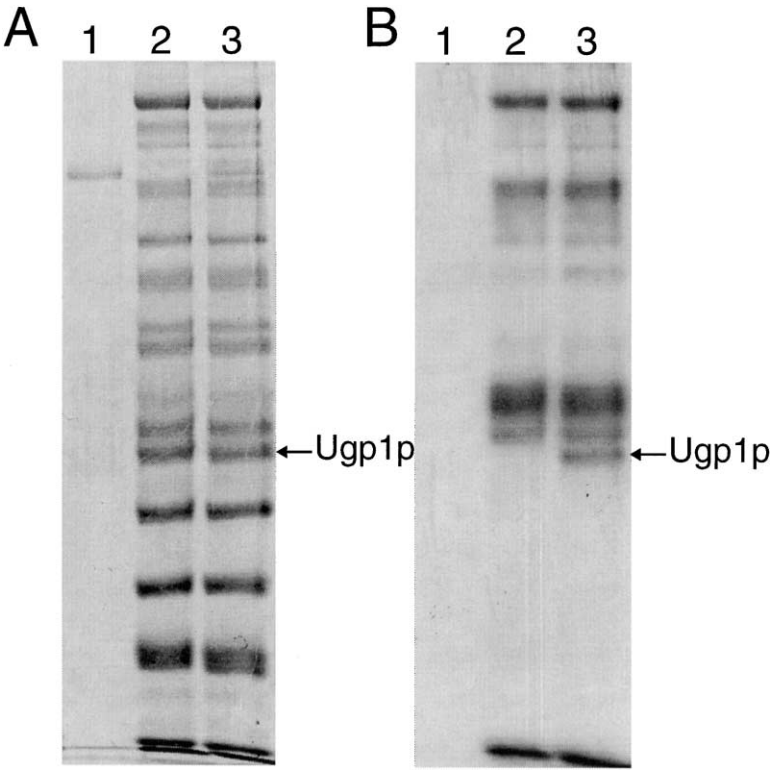

Figure 3. Biochemical Screen for Psk2p Substrates

Kinase reactions containing [γ-^{32}P]ATP were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). *S. cerevisiae* soluble lysate was passed through Ni^{2+}-NTA resin. Bound proteins were eluted by washing resin with buffer containing 250 mM imidazole. Lane 1: Psk2p alone; lane 2: Ni^{2+}-NTA elution alone; lane 3: Psk2p + Ni^{2+}-NTA elution.

interconversion of glucose-1-P and UTP with UDP-glucose and pyrophosphate (Figure 8). We cloned and expressed Ugp1p as a His_6-fusion in *E. coli* and found the purified protein to be efficiently phosphorylated by Psk2p (Figure 4, lanes 1–3). The site of Psk2p-mediated phosphorylation of Ugp1p was determined to be serine 11. A variant of Ugp1p wherein serine 11 was mutated to alanine (S11A) was unable to be efficiently phosphorylated by Psk2p (Figure 4, compare lanes 3–4).

We subsequently identified three additional proteins using this strategy. The second putative Psk2p substrate is the product of the CAF20 (*C*ap-*A*ssociated *F*actor 20) gene (de la Cruz et al., 1997). Eukaryotic translation initiation factor 4E (eIF4E) was also present in the fraction in which we identified Caf20p. These two proteins have been shown to constitute a stable complex upon purification to homogeneity (Altmann et al., 1989; Altmann and Trachsel, 1989). We cloned and expressed both eIF4E and Caf20p in bacteria. When assayed individually with Psk2p and [γ-^{32}P]ATP, neither protein was efficiently phosphorylated. By contrast, the Caf20p protein, when coexpressed and purified with eIF4E, was efficiently phosphorylated by Psk2p (Figure 4, lanes 5–7). We found two distinct sites of phosphorylation in Caf20p; one in the predominantly hydrophilic C-terminal 63 amino acids and the second in a region including serine residues 58 and 59. Both of the latter serine residues can be phosphorylated by Psk2p in a mutually exclusive manner. Either single mutant (S58A or S59A) was phosphorylated as efficiently as the native protein, but the S58A/S59A double mutant was immune to Psk2p-mediated phosphorylation in this region (Figure 4, lanes 7–9).

The third substrate identified in this biochemical screen is the product of the TIF11 gene, which encodes eukaryotic translation initiation factor 1A (eIF1A). eIF1A mediates the transfer of Met-tRNA to the 40S ribosomal subunit generating the 40S preinitiation complex (Chaudhuri et al., 1997). Upon expression and purification from *E. coli* as a His_6-fusion, Tif11p was phosphorylated by Psk2p (Figure 4, lanes 10–12). We mapped the site of phosphorylation to serine 125, which lies just C-terminal to the canonical eIF1A fold (Battiste et al., 2000). A variant of Tif11p lacking this residue (S125A) was immune to phosphorylation by Psk2p (Figure 4, lanes 12–13).

The fourth Psk2p substrate identified by these studies was the product of the SRO9 gene. Sro9p binds RNA in vitro, interacts with translating ribosomes, and deletion of SRO9 confers resistance to certain chemical inhibitors of translation (Sobel and Wolin, 1999). Attempts to express and purify ample quantities of full-length Sro9p from *E. coli* were unsuccessful. We found, however, that an N-terminally deleted form (Sro9pΔN55) was amenable for purification from bacterial lysate. Sro9pΔN55 was phosphorylated to the same level by Psk2p as full-length Sro9p (Figure 4, lanes 14–16). We mapped the site of phosphorylation to a tryptic peptide containing threonine residues 101 and 103. Both single mutants (T101A and T103A) were phosphorylated similarly to the wild-type protein, but the double mutant was immune to Psk2p-mediated phosphorylation (Figure 4, lanes 16–17). As observed for phosphorylation of Caf20p, Sro9p appears to be phosphorylated by Psk2p in a mutually exclusive manner at one of two adjacent residues.

Genetic Relationship of PSK2 and UGP1

UDP-glucose pyrophosphorylase (Ugp1p) catalyzes the generation of UDP-glucose and pyrophosphate from

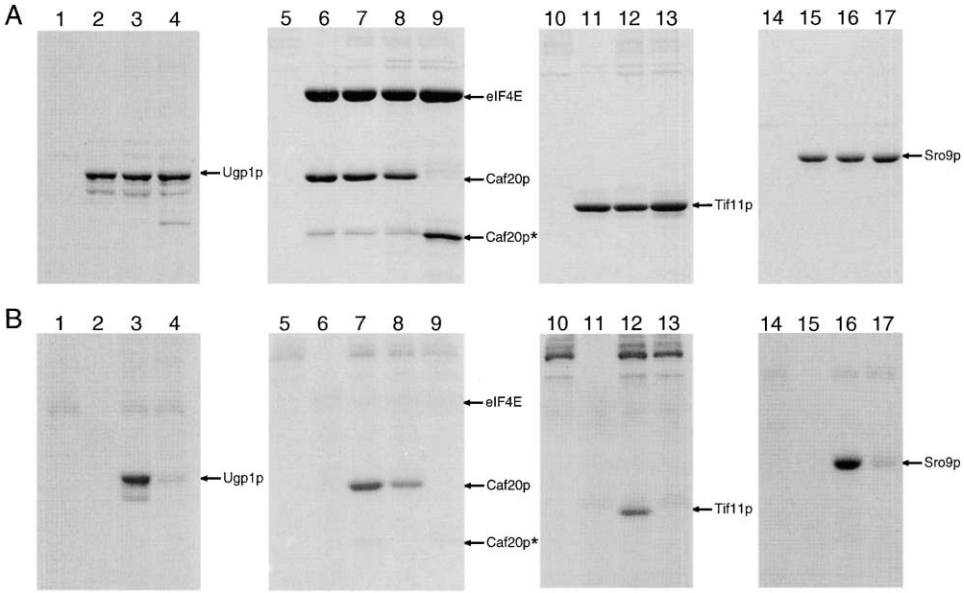

Figure 4. Psk2p Phosphorylates Ugp1p, Caf20p, Tif11p, and Sro9p

Kinase reactions containing [γ-^{32}P]ATP were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). Lanes 1, 5, 10, and 14: Pks2p alone; lanes 2, 6, 11, and 15: substrate alone; lanes 3, 7, 12, and 16: Psk2p + substrate; lanes 4, 8, 9, 13, and 17: Psk2p + mutant substrate. Substrate proteins are as follows: lanes 2 and 3: His_6-Ugp1p; lane 4: His_6-Ugp1p S11A; lanes 6 and 7: GST-eIF4E•His_6-Caf20p; lane 8: GST-eIF4E•His_6-Caf20p S58A/S59A; lane 9: GST-eIF4E•His_6-Caf20p S58A/S59A/ΔC63; lanes 11 and 12: His_6-Tif11p; lane 13: His_6-Tif11p S125A; lanes 15 and 16: His_6-Sro9pΔN55; lane 17: His_6-Sro9p T101A/T103A/ΔN55. Caf20p* signifies a naturally occurring C-terminally truncated form of Caf20p in lanes 6–8 and the Caf20pΔC63 deletion mutant in lane 9.

glucose-1-P and UTP (Figure 8). Due to the requirement for UDP-glucose in cell wall biosynthesis and protein glycosylation, Ugp1p is essential for viability of yeast (Daran et al., 1997). Overexpression of UGP1 is, however, detrimental to vegetative growth when *S. cerevisiae* is cultured on medium containing galactose as the carbon source (Daran et al., 1995). If PAS kinase were to phosphorylate and thereby regulate Ugp1p activity in vivo, then the effect of UGP1 overexpression should either be exacerbated or abrogated in a psk2 mutant strain (depending on whether PAS kinase-mediated phosphorylation activates or inactivates Ugp1p). We initially confirmed that overexpression of UGP1 (2 μ-UGP1) causes a modest impediment to vegetative growth on galactose-containing medium in the parental strain (Figure 5, top row). Deletion of the two PSK genes caused a marked increase in sensitivity to UGP1 overexpression, which was largely accounted for by the loss of the PSK2 gene (Figure 5, rows 2 and 4). Ugp1p enzymatic activity was strictly required for these adverse effects. Overexpression of an enzyme containing a G112D missense mutation, which eliminates more than 95% of enzymatic activity (Floresdiaz et al., 1997), exerted no effect on the growth of either a wild-type or any of the three psk mutant strains (Figure 5, third image).

We speculated that the hypersensitivity of the psk2 and psk1 psk2 mutant strains to UGP1 overexpression was due to an inability of these strains to phosphorylate serine 11 of Ugp1p. If so, a mutant of Ugp1p insensitive to PAS kinase-mediated phosphorylation (S11A) should be similarly toxic to the wild-type and three psk mutant strains. As shown in Figure 5, wild-type and the three psk mutant strains were equally sensitive to overexpression of UGP1 S11A as the psk1 psk2 double mutant was

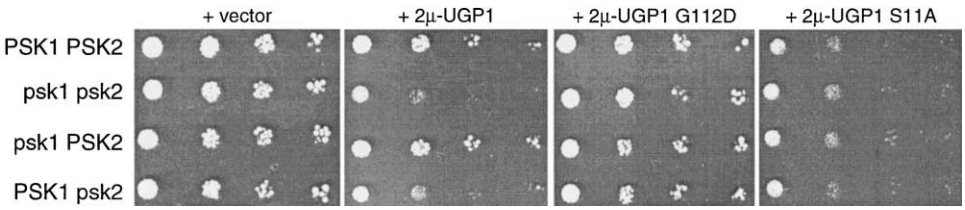

Figure 5. psk2 Mutants Are Hypersensitive to UGP1 Overexpression

Strains of the genotype indicated on the left were transformed with empty pRS424 (2μ TRP1) (first image), pRS424-UGP1 (second image), pRS424-UGP1 G112D (third image), and pRS424-UGP1 S11A (fourth image). Transformants were grown to saturation in minimal medium lacking uracil and tryptophan with 2% glucose at 30°C and diluted in water. These diluted samples were spotted at 10,000, 2000, 400, and 80 cells per spot onto minimal medium plates lacking uracil and tryptophan with 2% galactose. The plates were incubated at 30°C for 3 days. The strains shown are from top to bottom: parental (BHY10 + pRS416), psk double mutant (JRY40 + pRS416), psk1 single mutant (JRY40 + pRS416-PSK2), and psk2 single mutant (JRY40 + pRS416-PSK1).

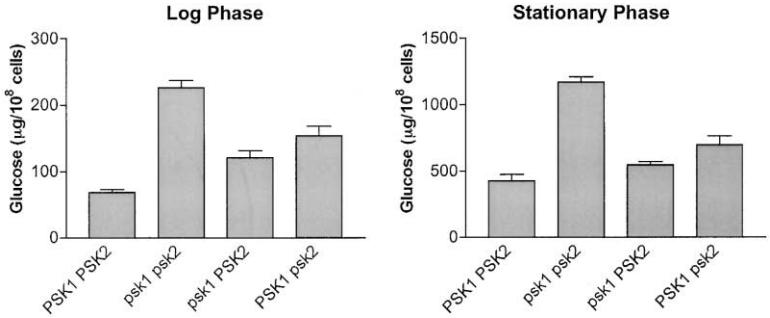

Figure 6. psk Mutant Yeast Accumulate Excess Glycogen

Strains of the indicated genotype were grown to saturation in minimal medium with 2% glucose at 30°C. Aliquots were harvested and assayed for glycogen content (right image). An aliquot of the saturated culture was diluted in fresh medium to an $OD_{600} = 0.1$ and cultured for 6 hr at 30°C to an $OD_{600} = 0.6$. An aliquot of this culture was harvested and assayed for glycogen content (left image) which is expressed as a concentration of glucose enzymatically released from glycogen. The mean of three experiments ± S.D. is shown. Note the difference in *y* axis scale in the two s. The strains shown are from left to right: parental (BHY10 + pRS416), psk double mutant (JRY40 + pRS416), psk1 single mutant (JRY40 + pRS416-PSK2), and psk2 single mutant (JRY40 + pRS416-PSK1).

to overexpression of wild-type UGP1 (Figure 5, fourth image). Apparently, both methods of blocking phosphorylation of serine 11 by Psk, deletion of the two PSK genes or mutation of the substrate, lead to indistinguishable growth defects.

PSK Mutants Accumulate Excess Storage Carbohydrate

The Ugp1p enzyme catalyzes an essential step in the synthesis of storage carbohydrates. As such, dysregulation of this enzyme might lead to inappropriate accumulation of the two major storage carbohydrates in yeast, glycogen and trehalose (Francois and Parrou, 2001). Indeed, underexpression of UGP1 leads to glycogen hypoaccumulation (Daran et al., 1997), and overexpression of UGP1 leads to hyperaccumulation of glycogen (Daran et al., 1995). We reasoned that if Psk were to phosphorylate and negatively regulate Ugp1, deletion of both PSK genes might cause increased storage carbohydrate synthesis. Indeed, psk1 psk2 double mutants accumulated 3- to 4-fold more glycogen than the parental strain in both log and stationary phase cultures grown in minimal glucose medium (Figure 6). Single psk mutants exhibited an intermediate glycogen hyperaccumulation phenotype, with the psk2 mutant being more affected than the psk1 mutant. In log phase cultures, similar results were obtained with the second major storage carbohydrate, trehalose. The psk1 psk2 double mutant, that is, accumulated elevated trehalose relative to the parental strain. By contrast, in stationary cultures parental and psk mutant strains accumulated essentially equivalent trehalose stores (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/111/1/17/DC1).

Two observations indicated that the storage carbohydrate hyperaccumulation phenotype evident in psk1 psk2 double mutants was not solely due to dysregulation of the Ugp1p enzyme. First, an artificially induced 40-fold increase in Ugp1p enzyme activity causes only a 2-fold increase in glycogen content (Daran et al., 1995). Deletion of the two PSK genes, comparatively, caused a reproducible 3- to 4-fold increase in glycogen content. Second, glycogen and trehalose are synthesized by the polymerization (glycogen) or dimerization (trehalose) of glucose from UDP-glucose. To the extent that carbohydrate storage hyperaccumulation is due to an increase in Ugp1p activity and concomitant increase in UDP-glucose, glycogen and trehalose storage should be equally affected. Whereas glycogen and trehalose levels were equally affected by psk mutation during exponential growth, only glycogen was increased during stationary phase. We therefore hypothesized that disruption of at least one additional regulatory process must contribute to glycogen hyperaccumulation in the psk1 psk2 double-mutant strain.

The two gene products that most directly control glycogen accumulation are glycogen synthase (the enzyme that adds glucose monomers to glycogen), and glycogen phosphorylase (the enzyme that removes glucose-1-P from glycogen) (Figure 8). Phosphorylation of either enzyme causes a net decrease in glycogen synthesis by inhibiting glycogen synthase (Hardy and Roach, 1993; Rothman-Denes and Cabib, 1971) and activating glycogen phosphorylase (Lin et al., 1995, 1996). The psk1 psk2 double-mutant strain has the phenotype, glycogen hyperaccumulation, predictive of diminished activity of either a glycogen synthase kinase or glycogen phosphorylase kinase. As such, we expressed and purified yeast glycogen synthase (Gsy2p) and glycogen phosphorylase (Gph1p) and assayed each enzyme for phosphorylation by Psk2p. Psk2p did not phosphorylate Gph1p (Figures 7A and 7B, lanes 1–3), but efficiently phosphorylated Gsy2p (Figures 7A and 7B, lanes 4–6). We mapped the site of phosphorylation to serine 654 (Figures 7A and 7B, lanes 6–7), which is one of three residues (also S650 and T667) previously shown to confer phosphorylation-mediated inhibition of glycogen synthase (Hardy and Roach, 1993).

The posttranslational regulation of glycogen synthase involves at least two processes: inactivation by phosphorylation of the three residues mentioned above and allosteric activation by glucose-6-P (Huang and Cabib, 1974a, 1974b). These two steps are interrelated, as the inactivation conferred by phosphorylation can be completely reversed by saturating glucose-6-P (Roach and Larner, 1976; Hardy and Roach, 1993; Rothman-Denes and Cabib, 1971). Therefore, glycogen synthase measurements in the presence of glucose-6-P are indicative of total enzyme concentration independent of phosphorylation state. Glycogen synthase activity can be expressed as the ratio of activity in the absence of glucose-6-P to activity in the presence of glucose-6-P (−/+ G6P), which is inversely related to phosphorylation state and independent of total enzyme concentration.

Extracts from exponentially growing wild-type and

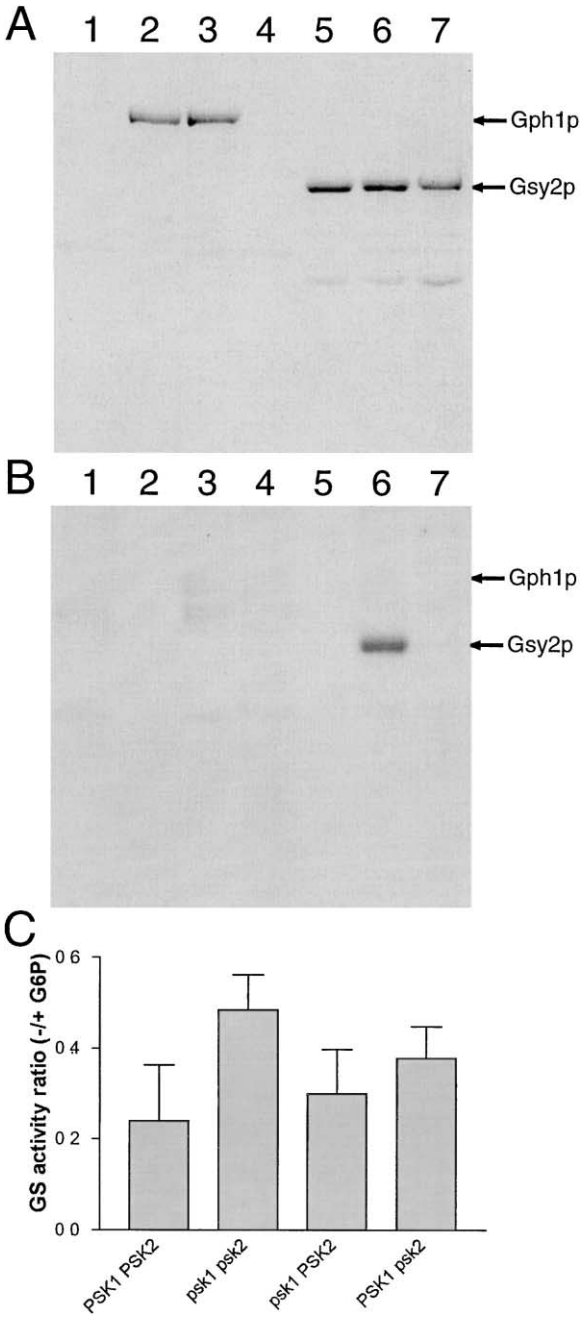

Figure 7. Glycogen Synthase Is Regulated by PAS Kinase-Mediated Phosphorylation

Kinase reactions containing [γ-^{32}P]ATP were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). Lanes 1 and 4: Psk2p alone; lane 2: Gph1p alone; lane 3: Psk2p + Gph1p; lane 5: Gsy2p alone; lane 6 Psk2p + Gsy2p; lane 7: Psk2p + Gsy2p S654A.

(C) Strains of the indicated genotype were grown to an OD_{600} = 1.7 in minimal medium with 2% glucose at 30°C. Cells were harvested, extracted, and assayed for glycogen synthase activity in the presence and absence of 6.7 mM glucose-6-P. The ratio of activity in the absence of G-6-P to activity in the presence of G-6-P (−/+ G6P) is displayed. The mean of three experiments ± S.D. is shown and is representative of eight independent experiments. The strains shown are from left to right: BHY10 + pRS416, JRY40 + pRS416, JRY40 + pRS416-PSK2, and JRY40 + pRS416-PSK1.

psk mutant strains contained similar glycogen synthase activity with and without glucose-6-P (data not shown). The depletion of nutrients in the medium (OD_{600} = 1.5 in minimal medium), however, correlated with the advent of a higher glycogen synthase activity ratio in the psk1 psk2 strain relative to wild-type (Figure 7C). The psk1 psk2 double mutants further retained an increased activity ratio through mid-stationary phase (OD_{600} = 2.5). As described above, such an increase in activity ratio is indicative of glycogen synthase being hypophosphorylated in the psk1 psk2 mutant strain. Similarly, mutation of the Psk phosphorylation site, serine 654, to alanine is known to cause an increase in the glycogen synthase activity ratio (Hardy and Roach, 1993). Thus, as demonstrated for UGP1, two independent methods of blocking phosphorylation of glycogen synthase by Psk, deletion of the PSK1 and PSK2 genes and mutational elimination of the Psk phosphorylation site, elicit similar phenotypic effects.

Discussion

Here we describe the use of two approaches aimed toward an assessment of the biological role of the two paralogous genes encoding the PAS kinase enzymes of *S. cerevisiae*. The first approach entailed yeast genetics, wherein cells of the psk1 psk2 double-mutant genotype were observed to grow poorly at elevated temperature when limited to galactose as the sole carbon source. We did not initially assume that the gal^{ts} phenotype would necessarily be relevant to the biological role of PAS kinase. The combined results of biochemical and genetic approaches indicate, however, that regulation of sugar flux may indeed represent one of the two roles of this enzyme.

Figure 8 summarizes the pathway by which *S. cerevisiae* employs galactose as an energy source. In the context of our understanding of this pathway, it is reasonably straightforward to interpret the mode of action of both high-copy suppressors and PAS kinase substrates. Under conditions of galactose utilization as the sole carbon source, it is essential that the sugar be converted through several steps into glucose-6-P in order to allow glycolytic production of ATP. In the absence of PAS kinase, we conclude that galactose falls into a shunt leading to the formation of storage carbohydrates. Overexpression of either of two paralogous phosphoglucomutase (Pgm) enzymes was found to suppress the gal^{ts} phenotype of the psk1 psk2 double mutant, consistent with the interpretation that the Pgm enzymes favor conversion of glucose-1-P into glucose-6-P, thereby pushing sugar flux toward glycolysis and pulling it away from the formation of storage carbohydrates.

We likewise report that two enzymes relevant to the control of sugar flux, UDP-glucose phosphorylase (Ugp1p) and glycogen synthase (Gsy2p), are direct targets of PAS kinase-mediated phosphorylation. As shown in Figure 8, the former enzyme catalyzes the conversion of glucose-1-P to UDP-glucose, which is one step removed from both glycogen and trehalose. The latter enzyme utilizes UDP-glucose directly in the polymerization of glycogen. Yeast strains missing both PAS kinase paralogs accumulate abnormally high levels of

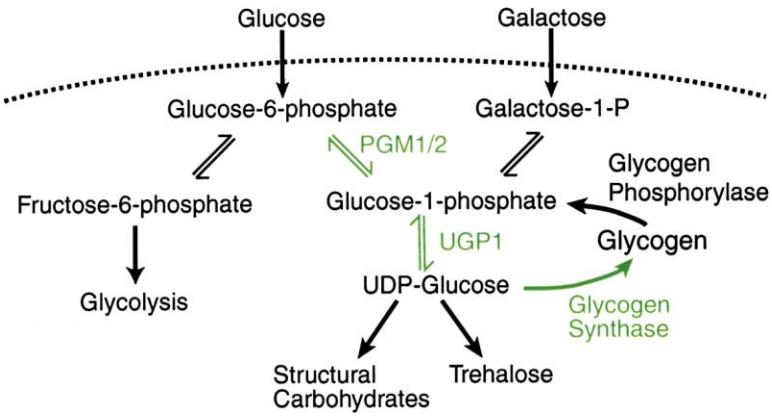

Figure 8. Model for Control of Metabolic Flux by Psk Enzymes

A part of the carbohydrate metabolic network of *S. cerevisiae* is diagrammed. Upon import, galactose is phosphorylated and epimerized to glucose-1-phosphate, which can then be converted to either glucose-6-phosphate for use in glycolysis and ATP production or UDP-glucose for structural and storage carbohydrate synthesis. Similarly, when glucose is imported into the cell, it is phosphorylated and can either be isomerized to fructose-6-phosphate for glycolysis, or converted to glucose-1-phosphate. The three enzymes catalyzing sequential steps in the incorporation of glucose into glycogen (shown in green) are all related to PAS kinase, two (UGP1 and glycogen synthase) representing direct substrates of the enzyme and another (PGM) representing a high-copy suppressor the psk1 psk2 double mutant.

glycogen and are interpreted to maintain both Ugp1p and Gsy2p in hypophosphorylated states favoring the formation of storage carbohydrates. We respectfully judge the evidence supportive of these interpretations to be compelling. The precise sites of Psk2p-mediated phosphorylation of Ugp1p and Gsy2p have been mapped and individually eliminated (Hardy and Roach, 1993). Directed mutation of the Psk2p phosphorylation sites of both Ugp1p and Gsy2p led to phenotypes similar to those observed in the psk1 psk2 double mutant. In other words, the same defects were observed irrespective of whether phosphorylation-mediated regulation was eliminated by removal of functional PAS kinase or mutational elimination of the precise site of phosphorylation. By negatively regulating both Ugp1p and Gsy2p, PAS kinase is interpreted to enhance the flux of galactose toward glycolysis, in part, by inhibiting the accumulation of glycogen.

The combined genetic and biochemical studies reported herein provide complimentary evidence favoring a second biological function of PAS kinase. In addition to its role in regulating sugar flux, PAS kinase may also serve to regulate protein synthesis. Three lines of evidence support a role for PAS kinase in the control of protein synthesis. First, the screen for high-copy suppressors of the psk1 psk2 double mutant identified a number of genes encoding translation factors and components of the translation apparatus (see Table 1). Of note is the previously uncharacterized RBS1 gene, which was the strongest suppressor identified. It contains an R3H domain that has also been implicated in translational regulation (Hawkins et al., 1997), and the integrity of the R3H domain was found to be essential for the activity of RBS1. Second, our unbiased screen for Psk2p substrates also led to the discovery of three polypeptides involved in the control of protein synthesis. Finally, high-copy expression of PAS kinase suppressed both the vegetative growth and protein synthesis deficits of a yeast strain lacking the translation initiation factor 4B (STM1).

It has previously been reported that the stm1 growth deficit is also suppressed by deletion of the CAF20 gene, which encodes one of the polypeptides identified as a Psk2p phosphorylation substrate (de la Cruz et al., 1997). The identification of Caf20p as a Psk2p substrate is of special note for three additional reasons. First, Caf20p negatively regulates translation by blocking the association of eIF4E and eIF4G (Altmann et al., 1997), which nucleates assembly of the translational apparatus at the 5′ cap of mRNAs (Hershey and Merrick, 2000). The DED1 and DBP1 genes, which encode highly similar DEAD-box RNA helicases involved in mRNA translation (Chuang et al., 1997), are genetically related to both eIF4E and CAF20. DED1 and DBP1 suppress the temperature-sensitive growth phenotype of an eIF4E mutant, and the ded1 mutant phenotype is partially suppressed by the deletion of CAF20 (de la Cruz et al., 1997). Since the DED1 and DBP1 genes were identified as suppressors of the psk1 psk2 double mutant, we offer the interpretation of a genetic link between CAF20 and the two PSK genes to compliment the biochemical link revealing Caf20p as a PAS kinase substrate.

A second reason to pay special note to Caf20p derives from studies of translational repression in higher eukaryotes. Caf20p likely represents the yeast analog of mammalian eIF4E binding proteins (4E-BPs). These proteins interact with eIF4E via a short peptide bearing significant sequence similarity to the eIF4E binding region of Caf20p (Raught et al., 2000). The mammalian 4E-BPs are an important convergence point for cellular signaling pathways that control translation. Insulin and other hormonal signals that lead to an increase in translation rate do so, at least in part, by triggering phosphorylation of 4E-BPs and catalyzing their release from eIF4E (Gingras et al., 1999; Lawrence and Abraham, 1997). Conversely, many stress conditions that lead to inhibition of translation cause hypophosphorylation of 4E-BPs, thereby enhancing their affinity for eIF4E (Raught et al., 2000; Zanchin and McCarthy, 1995).

A third notable point relating function of the CAF20 and PSK genes is that the phosphorylation state of 4E-BPs is closely coupled to nutrient availability. Glucose and amino acid withdrawal from cultured mammalian cells results in a decrease in the phosphorylation of 4E-BP1 (Hara et al., 1998). Organismal nutritional status, as signaled by insulin, also regulates 4E-BP1 (Gingras et al.,

1999). Cell autonomous nutrient signaling and hormonal signaling are coupled, with glucose and amino acid availability being permissive for insulin-stimulated 4E-BP1 phosphorylation (Hara et al., 1998; Patel et al., 2001; Xu et al., 1998). The pathways responsible for regulating Caf20p in *S. cerevisiae* are poorly understood. It is intriguing, however, that in a systematic effort to identify protein complexes in yeast, Ho and colleagues (Ho et al., 2002) found that Caf20p associates with a number of proteins involved in carbohydrate metabolism. This list includes Gal83p, a functional relative of the product of the SIP1 gene discovered in this study as a high-copy suppressor of the psk1 psk2 double mutant.

The results outlined herein show that the products of the PSK1 and PSK2 genes serve to coordinately regulate sugar flux and translation in the yeast, *S. cerevisiae*. The efforts of the present study have been focused upon the output pathway controlled by PAS kinase. By phosphorylating specific substrates involved in sugar flux and translation, PAS kinase may coordinate two pathways logically expected to act in synchrony. Since this enzyme contains one or more PAS domains that directly regulate the activity of its catalytic, serine-threonine kinase domain (Rutter et al., 2001), and since one of these PAS domains has now been shown to be capable of binding small chemical ligands (Amezcua et al., 2002), we hypothesize that this system has evolved to sense a specific metabolic product whose abundance is reflective of ambient nutritional state.

We close by comparing the proposed cell-autonomous role of PAS kinase to that of the insulin signaling system of mammals. In complex organisms, insulin acts as a humoral factor coordinating the storage/consumption of sugar (Lawrence and Roach, 1997) and efficiency of protein synthesis (Gingras et al., 1999; Lawrence and Abraham, 1997). Insulin action is gated by the nutritional status of the cells upon which it is destined to work. That is, if cells are deprived of either amino acids or glucose, they will not respond to insulin appropriately (Kimball and Jefferson, 2000; Patel et al., 2001). We tentatively speculate that PAS kinase, which is evolutionarily conserved from yeast to humans, may act in a cell-autonomous manner analogous to the humoral action of insulin.

Experimental Procedures

Strains, Media and Plasmids

S. cerevisiae strain BHY10 (MATα leu2-3,112::CPY-Inv LEU2 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2Δ9) (Horazdovsky et al., 1994) was obtained from B. Horazdovsky. Strain JRY10 (BHY10; psk2::NEO) was generated by homologous recombination, replacing the entire PSK2 ORF with the NEO gene. Strain JRY30 (BHY10; psk1::HIS3) was generated by replacing the entire PSK1 ORF with the HIS3 gene. Strain JRY40 (BHY10; psk1::HIS3 psk2::NEO) was generated by replacing the PSK2 gene with the NEO gene in strain JRY30. Integrity of recombination in mutants was confirmed by PCR and Southern blotting. *S. cerevisiae* strains CW04 (ade2 his3 leu2 trp1 ura3) and its derivative RCB1-1c (stm1::ADE2 ade2 his3 leu2 trp1 ura3) (Coppolecchia et al., 1993) were obtained from P. Linder. All medium was prepared as described (Sherman, 1991), and cultures were maintained at 30°C unless stated otherwise.

S. cerevisiae genes were amplified by PCR from genomic DNA derived from strain BHY10. For expression in yeast, genes were amplified with between 450 and 550 nucleotides of untranslated sequence both 5′ and 3′ to the ORF. Point mutants were generated by PCR-based mutagenesis. Plasmids maintained in yeast were derived from shuttle vectors pRS416 (CEN URA3) and pRS424 (2 μ TRP1) (Sikorski and Hieter, 1989). pRS416-PSK1 and pRS416-PSK2 were generated by cloning the relevant PSK gene into the SacI/XhoI or SpeI/XhoI sites of pRS416, respectively. pRS416-PSK2 ΔN792 was generated by PCR, fusing the sequence encoding residue #793 of Psk2p to the Psk2p initiator methionine and 400 bp of 5′ untranslated/promoter sequence. pRS424-UGP1 was generated by cloning the complete UGP1 gene into the SpeI and XhoI sites of pRS424.

Plasmids for bacterial expression of Ugp1p, Tif11p, Sro9p, Gsy2p, and Gph1p were created by cloning the relevant ORF into the plasmid pHis-Parallel (Sheffield et al., 1999) in-frame with the T7-promoter driven His_6 and TEV protease site sequence. The CAF20 ORF was first cloned into pHis-Parallel and the His_6/TEV site/CAF20 ORF cassette was subcloned into pOFX T7-1 (oriP15A kanR) (Castanie et al., 1997). The plasmid for expressing GST-eIF4E was created by cloning the CDC33 (eIF4E) ORF into pGST-Parallel (Sheffield et al., 1999) in-frame with the glutathione-S-transferase ORF and TEV protease site sequence.

Suppressor Screen

The *S. cerevisiae* genomic DNA library used for the high-copy suppressor screen was created by cloning partial Sau3AI-digested BHY10 genomic DNA into the BamHI site of pRS426 (2 μ URA3). Library-transformed JRY40 was plated onto minimal medium plates with 2% glucose lacking uracil at a density of 1000 colonies per plate, and incubated for 3 days at 30°C. The colonies from each plate were scraped into isolated pools and diluted in water to enable plating 1000 cells/plate on minimal medium lacking uracil with 2% galactose. These plates were then incubated at 39°C for 4 days. Viable colonies were propagated and their putative suppressing plasmid isolated and sequenced. Putative suppressing plasmids were confirmed by the same assay after transforming purified plasmid DNA into the JRY40 strain and testing under gal^{ts} conditions as described.

^{35}S-Labeling of Cultures

Strains of the indicated genotype were grown to log phase in minimal medium with 2% glucose. 0.5 OD of each culture was transferred to 37°C for 15 min, followed by addition of 2 μl [^{35}S]-methionine and cysteine (Promix, AmershamPharmacia). Labeling continued for 15 min at 37°C followed by glass bead lysis for 5 min in SDS-PAGE sample buffer. Samples were then boiled, separated by SDS-PAGE, fixed and exposed to autoradiographic film.

Kinase Substrate Screen, Assays, and Phosphorylation Site Mapping

The screen to identify kinase substrates utilized *S. cerevisiae* protein extracts generated as described (Buzan and Frieden, 1996). The extracts were applied to MonoQ, MonoS, Heparin Sepharose (all AmershamPharmacia), Bio-Gel hydroxyapatite (Bio-Rad), and Ni-NTA (Qiagen) chromatographic columns using an AmershamPharmacia FPLC system, followed by shallow gradient elution using conditions appropriate for each resin. The resulting fractions were subjected to a standard kinase assay using Psk2p under conditions identical to those described for human PASK (Rutter et al., 2001). After incubation for 30 min at 25°C, kinase reactions were terminated by the addition of SDS sample buffer and subjected to SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, dried, and analyzed by autoradiography. Proteins observed to be phosphorylated in a Psk2p-dependent manner were identified by mass spectrometry following in-gel trypsinolysis (Rosenfeld et al., 1992 as described by Vassilev et al., 2001). Kinase reactions using purified proteins were performed identically except that the fractionated extracts were replaced by the bacterial expressed and purified polypeptide. The in vitro substrates identified in this screen were isolated from the following fractions: Ugp1p—approximately 300mM NaCl elution from MonoQ following Ni-NTA agarose; Caf20p—approximately 350 mM NaCl elution from MonoQ following Ni-NTA agarose; Tif11p—approximately 400 mM NaCl elution from MonoQ of crude protein extract; Sro9p—approximately 250 mM NaCl elution from MonoQ following Ni-NTA agarose.

The phosphorylated residue for each substrate protein was identi-

fied by subjecting the protein to phosphorylation by Psk2p followed by SDS-PAGE and in-gel trypsinolysis (Hellman et al., 1995; Rosenfeld et al., 1992). Tryptic peptides were fractionated by reversed-phase HPLC on a Beckman ODS C_{18} column using a Beckman System Gold Model 126 HPLC system, eluting with a linear gradient of 0%–40% acetonitrile in 0.1%TFA/$H_2$0. Fractions (0.5 ml) were collected in deep well 96-well plates and subjected to scintillation counting using the Top Count Microplate Scintillation Counter (Packard Bioscience, Meriden, CT). The fraction corresponding to the peak of [^{32}P] radioactivity was subjected to mass spectroscopy as described (Vassilev et al., 2001). Experimentally determined masses were compared with the theoretical tryptic digest using the web-based MS-digest tool (http://prospector.ucsf.edu; Clauser et al., 1999). The identification of peptides by mass spectrometry was confirmed by automated Edman sequencing.

Protein Expression and Purification

Psk2p was expressed in and purified from Sf-9 cells infected with a recombinant baculovirus as described for human PASK (Rutter et al., 2001). All substrate proteins were expressed in *E. coli* strain BL21 (DE3) RIL (Stratagene). Ugp1p, Tif11p, Gsy2p, Gph1p, Sro9pΔN55, and Caf20p were expressed as His_6-tagged proteins and purified using Ni-NTA agarose (Qiagen) according to the manufacturers protocols. Ugp1p was further purified using MonoQ chromatography (AmershamPharmacia). Sro9pΔN55 was further purified using MonoQ and MonoS chromatography. The Caf20p•eIF4E complex was purified from *E. coli* cotransformed with the plasmids pOFX T7-His_6-Caf20 and pGST-Parallel-eIF4E. The induction conditions were as follows: Ugp1p, Gph1p and Sro9pΔN55-0.2 mM IPTG/24 hrs/25°C; Gsy2p-1 mM IPTG/14 hrs/30°C; Tif11p and Caf20p•eIF4E-0.2 mM IPTG/4 hrs/37°C.

Glycogen and Glycogen Synthase Assays

Glycogen content was assayed as described (Parrou and Francois, 1997). 20 OD of culture was quickly harvested, washed one time with H_2O, and frozen in liquid nitrogen. The thawed pellet was resuspended in 0.25 ml of 0.25 M Na_2CO_3 and incubated at 95°C for 4 hr, followed by addition of 0.15 ml of 1 M acetic acid and 0.65 ml of 0.2 M sodium acetate [pH 5.2]. Glycogen was hydrolyzed by *A. niger* amyloglucosidase (Sigma) (1.2 U/ml final) under constant shaking for 16 hr at 57°C, followed by centrifugation for 5 min at 14,000 g. The supernatant was assayed for glucose using the Sigma glucose assay kit (510-A).

Glycogen synthase activity was measured as described (Thomas et al., 1968), using extracts prepared essentially as described (Hardy et al., 1994), with the following exceptions. We used uridine diphospho-D-[6-^{3}H]glucose (specific activity of 0.1 mCi/mmol), and assayed for 20 min at 30°C. In addition, we passed the extracts over Microspin G-25 columns (AmershamPharmacia) prior to assay to remove low molecular weight molecules as described (Huang et al., 1997). One unit of activity is described as that sufficient to catalyze the transfer of 1 μmol of ^{3}H-glucose to glycogen per minute at 30°C. Assays were carried out both in the absence and in the presence of 6.7 mM Glucose-6-phosphate, and the resulting (−/+ G6P) glucose synthase activity ratio is often used as an indicator of glycogen synthase phosphorylation state.

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