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Yeast PAS kinase coordinates glucose partitioning in response to metabolic and cell integrity signaling

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PAS kinase is an evolutionarily conserved serine/threonine protein kinase. Mammalian PAS kinase is activated under nutrient replete conditions and is important for controlling metabolic rate and energy homeostasis. In yeast, PAS kinase acts to increase the synthesis of structural carbohydrate at the expense of storage carbohydrates through phosphorylation of the enzyme UDP-glucose pyrophosphorylase. We have identified two pathways that activate yeast PAS kinase; one is responsive to nutrient conditions while the other is responsive to cell integrity stress. These pathways differentially activate the two PAS kinase proteins in *Saccharomyces cerevisiae*, Psk1 and Psk2, with Psk1 alone responding to activation by non-fermentative carbon sources. We demonstrate that, in addition to transcriptional effects, both of these pathways post-translationally activate PAS kinase via its regulatory N-terminus. As a whole, this system acts to coordinate glucose partitioning with alterations in demand due to changes in environmental and nutrient conditions.

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Introduction

Organisms must adapt to an ever-changing environment, including cycles of nutrient availability and limitation. Therefore, cells have evolved complex mechanisms for sensing their nutritional environment and adjusting their metabolism appropriately. Failure to properly coordinate cellular metabolism results in a variety of human diseases and syndromes including diabetes and obesity (Lindsley and Rutter, 2004). Two of the best recognized nutrient-sensing protein kinases, AMPK and mTOR, have been implicated in the development of metabolic diseases and are promising therapeutic targets (Eurich *et al*, 2005; Fryer and Carling, 2005; Sarbassov dos *et al*, 2005). Mammalian AMPK senses cellular energy depletion and acts to increase cellular ATP content (Hardie *et al*, 1998). Mammalian TOR is activated by a variety of signals, including amino-acid availability, and promotes growth and proliferation (Schmelzle and Hall, 2000). PAS kinase, a serine/threonine protein kinase conserved from yeast to man, is a newly discovered member of this group of nutrient-sensing protein kinases (Rutter *et al*, 2001).

In addition to a canonical kinase catalytic domain, PAS kinase contains a regulatory PAS (Per-Arnt-Sim) domain that may provide insight into the mode of PAS kinase regulation. PAS domains have been shown to sense the intracellular environment by binding a small molecule ligand and regulating an attached functional domain in *cis* (Taylor and Zhulin, 1999). In PAS kinase, the PAS domain specifically interacts with and inactivates the kinase catalytic domain (Rutter *et al*, 2001; Amezcua *et al*, 2002). Based on biochemical data, we proposed that PAS kinase activity is regulated *in vivo* by PAS domain interaction with an unknown metabolite. Indeed, PAS kinase is activated by elevated glucose in cultured β-cells (da Silva Xavier *et al*, 2004), a condition resulting in increased mitochondrial metabolism.

In response to metabolic signals, mammalian PAS kinase acts to determine metabolic rate and energy homeostasis. PAS kinase is required for normal glucose-stimulated insulin production in cultured β-cells (da Silva Xavier *et al*, 2004) and for normal insulin secretion *in vivo* (Hao *et al*, 2007). Mice lacking PAS kinase also exhibit resistance to the obesity and insulin resistance caused by a high-fat diet (Hao *et al*, 2007). This is likely due to increased metabolic rate in peripheral tissues, including skeletal muscle. Thus, mammalian PAS kinase is directly involved in sensing and responding to metabolic status.

Yeast PAS kinase also regulates glucose partitioning through phosphorylation of the enzyme UDP-glucose pyrophosphorylase (Ugp1) (Smith and Rutter, 2007). Ugp1 produces UDP-glucose, the immediate glucose donor for both glycogen and cell wall glucan biosynthesis. Surprisingly, phosphorylation of Ugp1 by PAS kinase does not change the catalytic activity of Ugp1, but instead alters the subcellular location of the Ugp1 enzyme and thereby the destination of its product UDP-glucose. PAS kinase-deficient yeast, as well as yeast containing an unphosphorylatable mutant of Ugp1, have increased glycogen content and decreased cell wall glucan content (Smith and Rutter, 2007). Therefore, PAS kinase-dependent phosphorylation of Ugp1 controls the ultimate fate of glucose by stimulating cell wall glucan biosynthesis at the expense of glycogen synthesis. Here, we demonstrate that PAS kinase activity is regulated by metabolic status and cell integrity stress, making yeast PAS kinase a sensory integrator that coordinates the fate of intracellular glucose with metabolic demand.

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Results

Cell integrity stress activates PAS kinase-dependent phosphorylation of Ugp1

The biological function of the phosphorylation of Ugp1 by PAS kinase has recently been described (Smith and Rutter, 2007). The phosphorylation of Ugp1 by PAS kinase does not change Ugp1 catalytic activity but causes a conformational transition that may be detected using ion-exchange chromatography. That is, unphosphorylated and phosphorylated forms of Ugp1 elute as distinct species that may be independently quantified. We have employed this phenomenon to measure the *in vivo* phosphorylation of Ugp1 as an indicator of PAS kinase activity.

Because it stimulates cell wall glucan synthesis, we hypothesized that PAS kinase might be activated under conditions of increased cell wall demand, such as cell integrity stress. Many stimuli, including sodium dodecyl sulfate (SDS) are known to elicit cell integrity stress (Bickle *et al*, 1998). As shown in Figure 1, treatment with SDS enhanced PAS kinase-dependent phosphorylation of Ugp1 in a dose-dependent manner. To confirm cell integrity stress-specific activation of PAS kinase, the effects of various compounds known to distinctively elicit cell integrity stress were assessed (Figure 1B). Ugp1 phosphorylation was not only stimulated by cell wall-perturbing agents, like calcofluor white, but also by chlorpromazine, which specifically perturbs membrane structure (Kamada *et al*, 1995). These stressors are known to activate the canonical cell integrity pathway, which consists of an upstream family of stress sensors, the Wsc proteins, which activate a signaling cascade, including Rho1 and MAP kinase (Sekiya-Kawasaki *et al*, 2002). PAS kinase appears to also be activated by the Wsc family. Overexpression of Wsc1, the predominant member of this family (Verna *et al*, 1997; Rajavel *et al*, 1999), causes activation of PAS kinase even in the absence of exogenous stressors (Figure 1C). However, PAS kinase activation in response to SDS occurred almost normally in a *wsc1* deletion (data not shown). We hypothesize that this is due to the presence of a large and highly redundant family of Wsc1-related proteins in *Saccharomyces cerevisiae* (Verna *et al*, 1997; Rajavel *et al*, 1999). PAS kinase activation in response to SDS also occurred normally in deletions of other cell integrity pathway genes, including *wsc2*, *wsc3*, *mpk1*, *mid2*, *mtl1*, *msb1*, *lre1*, *rom2*, *tus1* and *zds1* (data not shown). In addition, overexpression of *PSK2* did not rescue the growth phenotypes of cell integrity pathway mutants, including *wsc1*, *mpk1*, *mtl1*, *rom2* and *zds1*. We hypothesize that PAS kinase is activated by the upstream cell integrity pathway sensors, the Wsc family,

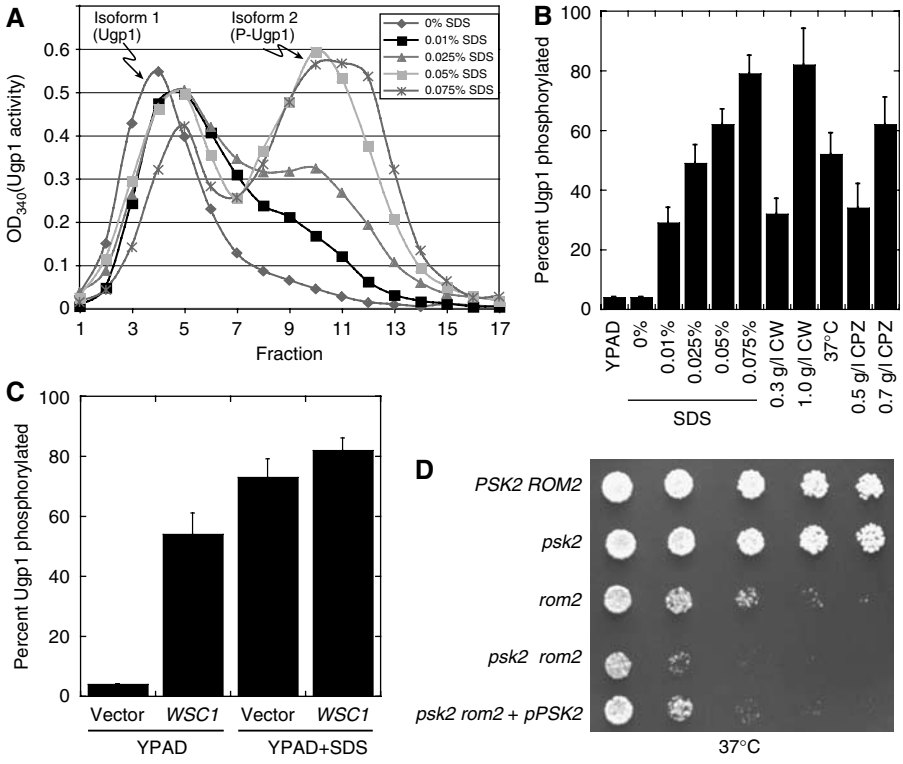

Figure 1 Cell integrity stress activates PAS kinase-dependent phosphorylation of Ugp1. (**A**) Ugp1 activity in fractions of crude yeast extracts separated by MonoQ. Wild-type cells (JRY 245) were treated with the concentration of SDS indicated for 2 h. (**B**) Quantification of the percent phosphorylation of Ugp1 from cells treated as indicated. Cells were grown in triplicate to an OD_{600} of 0.6 before treatment with SDS, calcofluor white (CW), high temperature (37°C) or chlorpromazine (CPZ) for 2 h. Cells were assayed for Ugp1-phosphorylation and the percent of phosphorylated Ugp1 (±s.d.) is shown. (**C**) Wsc1 overexpression is sufficient to activate PAS kinase. The wild-type strain (JRY245) containing either pRS426 (vector) or pRS426-*WSC1* (*WSC1*) was grown to saturation in SD-uracil medium and then diluted and grown in YPAD in triplicate to an OD_{600} of 0.6 before harvest (YPAD) or treatment with SDS for 2 h (YPAD + SDS). Cells were assayed for Ugp1-phosphorylation and the percent of phosphorylated Ugp1 (±s.d.) is shown. (**D**) Deletion of *PSK2* exacerbates the temperature sensitive growth phenotype of a *rom2* deletion mutant. Strains of the indicated genotype were grown to saturation and serially diluted in water. These diluted samples were spotted onto minimal glucose medium lacking uracil, and incubated at 37°C for 3 days. The strains used were JRY245 (wild type + pRS416), JRY853 (*psk2 ROM2* + pRS416), JRY854 (*PSK2 rom2* + pRS416), JRY858 (*rom2 psk2* + pRS416) and JRY858 (*rom2 psk2* + pRS416-PSK2).

but functions independently of the downstream components of this pathway to confer cellular stability. Consistently, a *psk2* deletion exacerbates the temperature-sensitive growth phenotype of a mutant lacking the Rho1 guanine nucleotide exchange factor ROM2 (Figure 1D).

To summarize, cell integrity stress stimulated PAS kinase-dependent phosphorylation of Ugp1, which favors the biosynthesis of cell wall glucans necessary for repair. The failure of this compensatory response is likely to cause the growth defect of a *psk1 psk2* mutant under conditions of cell integrity stress (Smith and Rutter, 2007).

Metabolic status regulates PAS kinase-dependent phosphorylation of Ugp1

Based on the metabolic sensing function of mammalian PAS kinase, we examined yeast PAS kinase activity under a variety of nutrient conditions. We observed no change in Ugp1 phosphorylation when cells were grown under a range of amino acid or nitrogen concentrations (data not shown). We did, however, observe increased Ugp1 phosphorylation in yeast grown in carbon sources other than glucose (Figure 2A). When *S. cerevisiae* is cultured in glucose, they primarily ferment the glucose to ethanol and repress the genes required for growth in less-optimal nonfermentative carbon sources (such as ethanol and glycerol). This phenomenon is known as glucose repression (Carlson, 1999). Glucose derepression requires the AMPK ortholog Snf1. It occurs in nonfermentative carbon sources, when glucose is at low concentrations, or when Snf1 is hyperactivated, such as in strains lacking Reg1. Reg1 is required for Snf1 dephosphorylation and inactivation (Bisson, 1988; Tu and Carlson, 1995; Sanz *et al*, 2000). Elimination of the glucose repression system by deleting *REG1* caused full PAS kinase activation in glucose medium (Figure 2B). As expected, PAS kinase activation in the *reg1* mutant required functional Snf1. Conversely, activation of PAS kinase by cell integrity stress was unaffected by deletion of the *SNF1* gene (Figure 2B).

These data support a model wherein two independent signals, a metabolic signal involving Snf1 and cell integrity stress involving the Wsc proteins, converge on PAS kinase, causing its activation and subsequent partitioning of glucose toward glucan biosynthesis at the expense of glycogen biosynthesis. As predicted by this model, glycogen levels are threefold higher in the *psk1 psk2* mutant relative to the wild-type strain when grown under either activating condition. However, when cell integrity stress is prevented in glucose medium, the glycogen levels are identical in the wild-type and *psk1 psk2* mutant strain (Supplementary Figure 1).

Many glucose-repressed genes are involved in allowing more efficient and complex metabolism, including mitochondrial biogenesis and activity. Thus, the PAS kinase activating stimuli in *S. cerevisiae* (nonfermentative carbon sources) and in cultured β-cells (elevated glucose) have a similar physiological consequence, increased mitochondrial metabolism. We hypothesize that the molecular signal linking metabolic status to PAS kinase activation is an evolutionarily conserved sentinel of mitochondrial activity.

Differential roles for Psk1 and Psk2 in response to metabolic status and cell integrity stress

The *S. cerevisiae* genome encodes two PAS kinase homologs, Psk1 and Psk2. They are highly similar in sequence and have been shown to be partially redundant in function (Rutter *et al*, 2002). We determined the ability of both Psk1 and Psk2 to individually phosphorylate Ugp1 in response to either the metabolic or cell integrity stimulus. The presence of either Psk1 or Psk2 was sufficient to cause Ugp1 phosphorylation in response to cell integrity stress, with Psk2 appearing to predominate (Figure 3A). Ugp1 phosphorylation in response to the metabolic stimulus (growth in raffinose instead of glucose), however, completely required Psk1. The absence of Psk1, even in the presence of the wild-type *PSK2* gene,

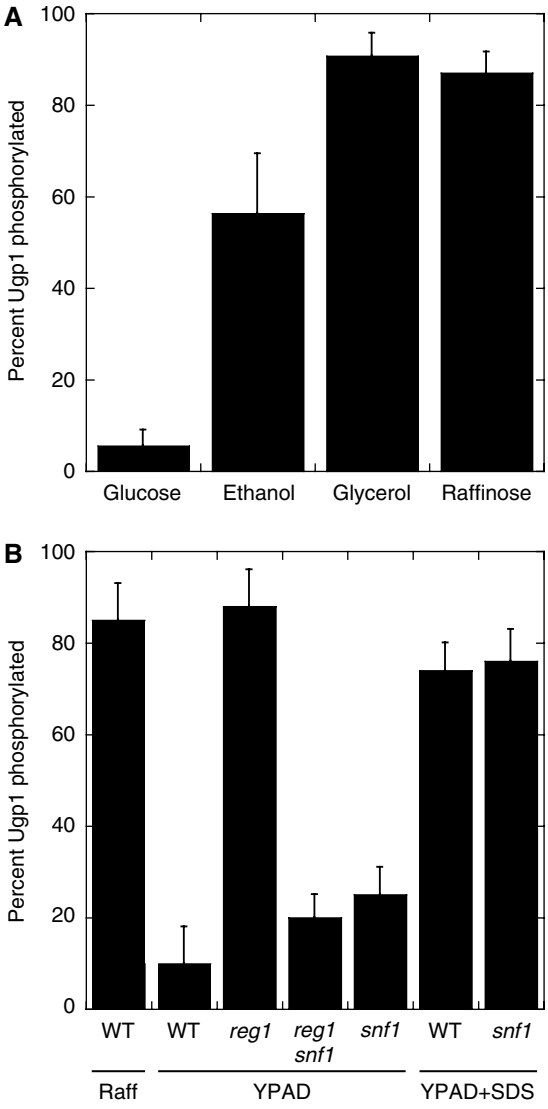

Figure 2 The glucose derepression pathway stimulates phosphorylation of Ugp1. (**A**) Nonfermentative carbon sources stimulate Ugp1 phosphorylation. Wild-type cells (JRY 245) were grown in triplicate to an OD_{600} of 0.6 in YPA with the carbon source indicated, were assayed for Ugp1-phosphorylation and the percent of phosphorylated Ugp1 (±s.d.) is shown. (**B**) Snf1 kinase activity is necessary and sufficient for PAS kinase-dependent Ugp1 phosphorylation in response to the metabolic stimulus, but not cell integrity stress. Cells of the indicated genotype were grown in triplicate in either YPAD or YPA-Raffinose (Raff) to an OD_{600} of 0.6 and then either harvested (YPAD and Raff) or subjected to 0.05% SDS for 2 h (YPAD + SDS). Cells were assayed for Ugp1 phosphorylation and percent Ugp1 phosphorylation (±s.d.) is displayed. The strains used were JRY245 (wild type), JRY456 (REG1 snf1) JRY506 (*reg1 SNF1*) and JRY514 (*reg1 snf1*).

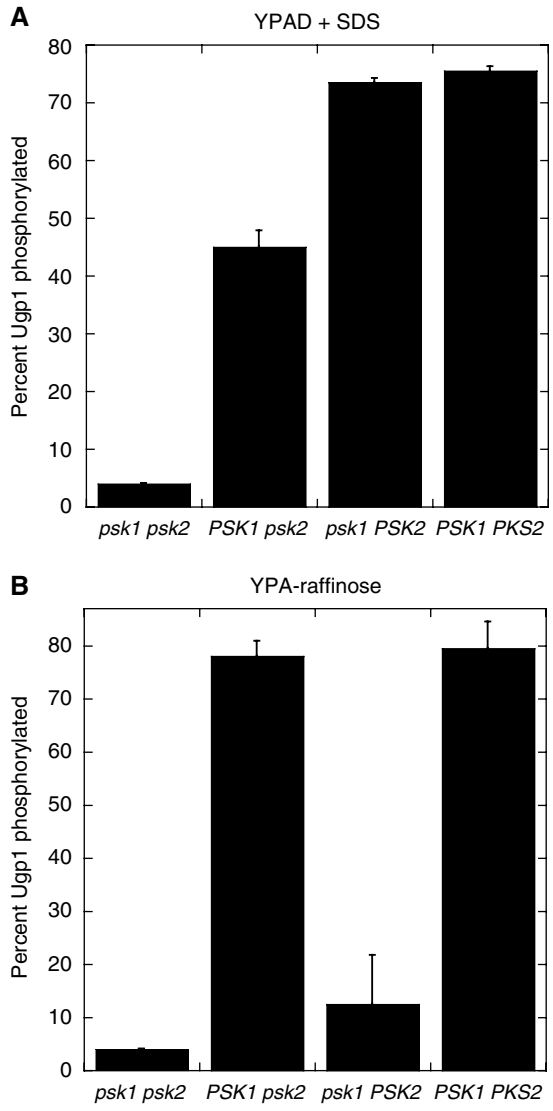

Figure 3 Differential roles for Psk1 and Psk2 in Ugp1 phosphorylation activated by cell integrity stress and nonfermentative carbon source. (**A**) Cells of the indicated genotype were grown in triplicate in YPAD to an OD_{600} of 0.6, treated for 2 h with 0.05% SDS and assayed for Ugp1 phosphorylation. Percent Ugp1 phosphorylation ($\pm$s.d.) is displayed. (**B**) Cells of the indicated genotype were grown in triplicate in YPA-Raffinose to an OD_{600} of 0.6 and assayed for Ugp1 phosphorylation. Percent Ugp1 phosphorylation ($\pm$s.d.) is displayed. The strains used were JRY245 (*PSK1 PSK2*), JRY276 (*psk1 PSK2*), JRY277 (*PSK1 psk2*) and JRY 278 (*psk1 psk2*).

caused an almost complete loss of this response (Figure 3B). The failure of Psk2 to phosphorylate Ugp1 in response to the metabolic stimulus in the *psk1 PSK2* strain appears to be due to a near complete loss of *PSK2* mRNA (data not shown). This is reflected in the protein levels of Psk2 under these conditions (Figure 4B).

Enhancement of PAS kinase enzymatic activity by the metabolic and cell integrity stimuli

We have used the *in vivo* phosphorylation state of Ugp1 as a marker of PAS kinase activity, however, other cellular components (such as a Ugp1-specific phosphatase) could be playing a role in determining the levels of phospho-Ugp1.

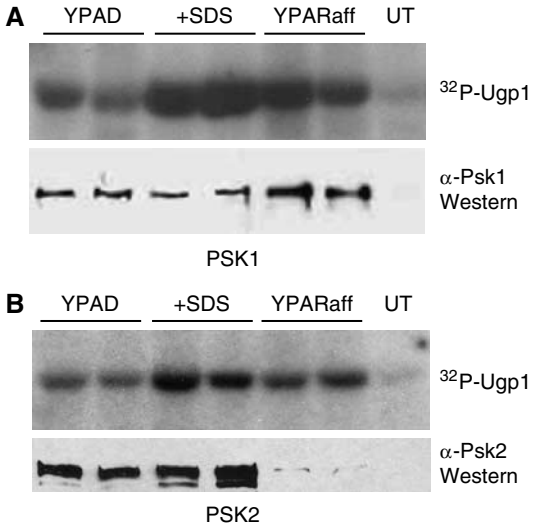

Figure 4 Activation of Psk1 and Psk2 by cell integrity stress and growth in nonfermentative carbon source. Kinase assay and western blot of immunoprecipitated Psk1 (**A**) or Psk2 (**B**). Cells expressing the Psk1-TAP and Psk2-TAP fusion proteins (or controls) were grown in either YPAD or YPA-Raffinose (YPARaff) to an OD_{600} of 0.6 and then either the harvested (YPAD and YPARaff) or the YPAD grown cells were subjected to 0.05% SDS for 2 h ($+$SDS). PAS kinase was immunoprecipitated and assayed for kinase activity using $\Upsilon[^{32}P]$ATP and Ugp1 as a substrate (upper panels). PAS kinase protein was visualized by western blotting (lower panels). Identically treated duplicates are shown for each condition. The strains used were JRY405 (*PSK1-TAP*::*kanMX4*) and JRY406 (*PSK2-TAP*::*kanMX4*).

Therefore, in addition to monitoring the *in vivo* level of phospho-Ugp1, we have measured PAS kinase activity directly using *in vitro* assays of immunoprecipitated PAS kinase with Ugp1 added as a substrate. PAS kinase proteins retained their appropriate activity state throughout the immunoprecipitation procedure (i.e., cells that displayed activated PAS kinase by monitoring phospho-Ugp1 levels yielded purified activated PAS kinase). Purified Psk1 and Psk2 from cells grown under conditions of cell integrity stress exhibited a four-fold increase in activity relative to the glucose-grown control, while protein levels remained relatively constant or even decreased slightly (Psk1) (Figure 4A, B, respectively). These results suggest that Psk1 and Psk2 are post-translationally activated in response to cell integrity stress.

Both Psk1 and Psk2 showed increased *in vitro* activity in response to the metabolic stimulus as well (Figure 4A, B, respectively). The interpretation of these results, however, is more complicated than the response to cell integrity stress because of changes in the levels of Psk1 and Psk2 proteins. First, the level of Psk1 protein increases slightly (Figure 4A). This increase is likely due to an increase in the *PSK1* mRNA in YPA-raffinose (data not shown). Second, Psk2 activity increases only slightly (approximately 25%), but this increased activity occurs in spite of a more than fivefold decrease in Psk2 protein (Figure 4B). As described previously, this decrease in Psk2 protein is likely due to decreased *PSK2* mRNA (data not shown). Although these data are consistent with Psk1 and Psk2 being post-translationally activated in response to metabolic status, they are insufficient to draw a firm conclusion.

Post-translational activation of PAS kinase by metabolic status and cell integrity stress

To more rigorously test whether PAS kinase is post-translationally activated by the metabolic stimulus and cell integrity stress, we eliminated changes in the expression of the PAS kinase genes using constructs that express Psk1 and Psk2 constitutively. This was accomplished by generating strains wherein Psk2 was expressed from one of the three different constitutive promoters, namely *CYC1Δ*, *CMD1* and *STE20*. As seen in Figure 5A, PAS kinase-dependent Ugp1 phosphorylation was stimulated by both SDS and raffinose in each of these strains. As expected, the activation of Psk2 by metabolic status was not seen with the endogenous Psk2 promoter, consistent with a loss of Psk2 expression in response to nonfermentative carbon source (see Figure 4B). We obtained almost identical results using constructs expressing Psk1 from these same three promoters (data not shown).

The CMD1 and STE20 promoters were used to generate constructs that constitutively express TAP-tagged forms of Psk1 and Psk2. These constructs were integrated into the native *PSK1* and *PSK2* loci and were used to evaluate the protein levels and *in vitro* activity of Psk1 and Psk2 under constitutive expression. Similar data were obtained using either of the promoters and both Psk1 and Psk2. For simplicity, only the data using the STE20 promoter–PSK1 fusion is presented. As expected, Psk1 activity was increased by both SDS and raffinose relative to YPAD conditions (Figure 5B). We observed similar Psk1 protein levels in all conditions. Thus, both the metabolic and cell integrity stimuli are able to activate PAS kinase in the absence of alterations in protein amount. To determine the role of the PAS kinase N-terminus (which includes the PAS sensory domain) in activation, we constructed a deletion mutant lacking the N-terminal 819 amino acids of Psk2 expressed from the STE20 promoter. This truncated protein was not activated in response to either SDS or raffinose (Figure 5C). The small apparent increase in activity is mirrored by a small increase in Psk2 protein under these conditions. We therefore conclude that metabolic and cell integrity stimuli act via the N-terminal regulatory region (and potentially the PAS domain) to activate PAS kinase and thereby alter glucose partitioning.

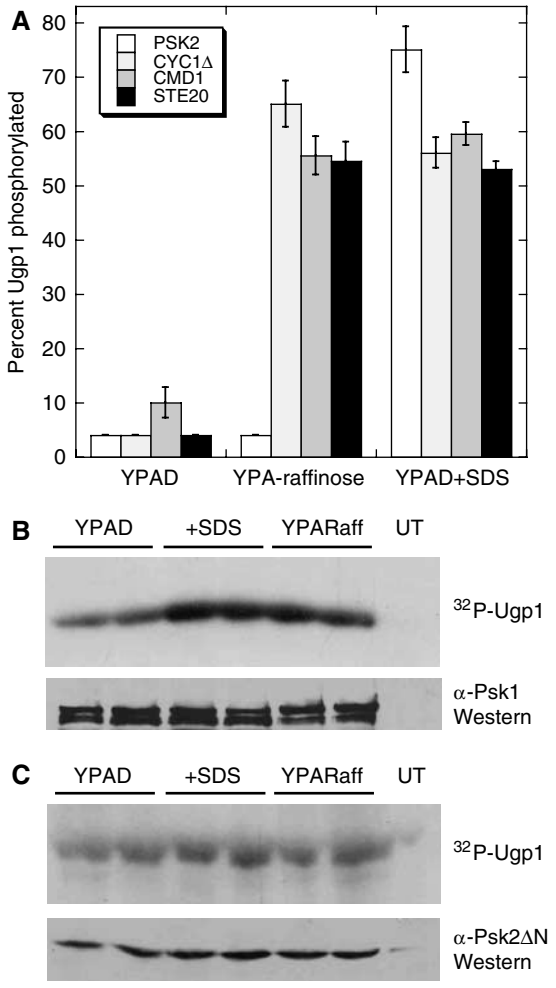

Figure 5 Activation of constitutively expressed Psk1 and Psk2 by cell integrity stress and growth in nonfermentative carbon source. (**A**) Strains wherein PSK2 was expressed under the control of each of four different promoters were grown to an OD_{600} of 0.6 in YPAD or YPA-Raffinose as indicated. They were then either harvested (YPAD and YPA-Raffinose) or subjected to 0.05% SDS for 2 h (YPAD + SDS). Cells were assayed for Ugp1 phosphorylation and percent Ugp1 phosphorylation (±s.d.) is displayed. (**B, C**) Kinase assay and western blot of immunoprecipitated constitutively expressed Psk1 (B) or Psk2ΔN (C), which lacks the N-terminal 819 residues of Psk2. The strain expressing a Psk1-TAP fusion (JRY684) (B) or Psk2ΔN-TAP fusion (JRY674) (C) from the STE20 promoter was grown to an OD_{600} of 0.6 and then either harvested (YPAD and YPARaff) or the YPAD samples were subjected to 0.05% SDS for 2 h (+SDS). PAS kinase was immunoprecipitated and assayed for kinase activity using $\Upsilon[^{32}P]$ATP and Ugp1 as a substrate (upper panel). PAS kinase protein was visualized by western blotting (lower panel). Identically treated duplicates are shown for each condition. The promoters and strains used are described in Materials and Methods.

Discussion

The ability of a cell to monitor its environment and respond accordingly is critical for survival. We have uncovered a mechanism by which cells can sense their environment and adjust their metabolism to correctly partition glucose either for storage or for growth and proliferation. PAS kinase, which we propose to be a major sensory and signaling component in this system, regulates the utilization of glucose to form glycogen or cell wall components through the phosphorylation of Ugp1. Phosphorylation of Ugp1 does not affect its activity, but rather alters the destination of its product UDPG, favoring glucan biosynthesis over glycogen biosynthesis (Smith and Rutter, 2007; Figure 6).

We have utilized both *in vivo* and *in vitro* assays to identify two stimuli that activate PAS kinase-dependent phosphorylation of Ugp1 (Figure 6). One activating stimulus is cell integrity stress, which can be elicited through any of a large number of manipulations. The Wsc family of membrane proteins are believed to act as sensors of membrane or cell wall damage and to initiate a stress-response pathway through the Rho1 small GTPase known as the cell wall integrity pathway (Levin, 2005). We have shown that overexpression of one member of the Wsc family, Wsc1, was sufficient to activate PAS kinase even in the absence of additional stressors. The Wsc1 protein, however, was not necessary for PAS kinase activation in response to cell integrity stress, likely a result of the redundancy within the Wsc family (Sekiya-Kawasaki *et al*, 2002). We also demonstrate that PAS kinase activation is independent of

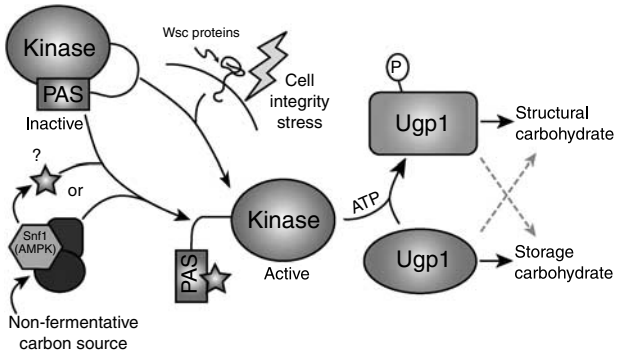

Figure 6 A model for PAS kinase activation by nonfermentative carbon source and cell integrity stress in *S. cerevisiae*. Autoinhibited PAS kinase can be activated by either of two signals. Nonfermentative carbon sources, acting via the Snf1 kinase complex, activate PAS kinase perhaps through the production of a PAS domain-binding metabolite (star). PAS kinase can also be activated by cell integrity stress acting through the Wsc protein family. Activated PAS kinase phosphorylates Ugp1, causing a conformational change in the protein, which leads to increased glucan synthesis (structural carbohydrate) at the expense of glycogen synthesis (storage carbohydrate). A full color version of this figure is available at the *EMBO Journal* Online.

downstream components of the cell wall integrity pathway, specifically the Rho1 guanine nucleotide exchange factors Rom2 and Tus1, the MAP kinase Mpk1 and others. Upon PAS kinase activation and Ugp1 phosphorylation, cell wall glucan synthesis is stimulated and the cell integrity apparatus is strengthened (Smith and Rutter, 2007). We believe that this is a cell integrity homeostatic system that detects cell wall insufficiency and signals to correct it through increased cell wall biosynthesis at the expense of glycogen biosynthesis.

The second stimulus shown to activate PAS kinase is metabolic in nature, growth on a nonfermentative carbon source. More specifically, we have shown that this PAS kinase activation pathway requires glucose derepression via the Snf1 kinase complex (Figure 6). One of the effects of Snf1 activation and glucose derepression is transcriptional induction of mitochondrial biogenesis and respiratory metabolism. It is unclear why it is advantageous for *S. cerevisiae* to increase glucan production under conditions of increased mitochondrial metabolism. It is possible that the cell interprets increased mitochondrial metabolic flux as a marker of nutrient sufficiency and impending cell division. During cell division, a yeast cell must completely regenerate its elaborate cell wall, a formidable task as the cell wall, primarily composed of glucans, accounts for up to 25% of the cell's dry weight (Klis *et al*, 2006). In this context, PAS kinase could be acting prospectively to prepare the cell for the upcoming division by increasing glucan production at the expense of glycogen. Thus, in response to both stimuli, PAS kinase acts through Ugp1 phosphorylation to correct a perceived glucan deficiency. In support of this model, glycogen levels are threefold higher in the *psk1 psk2* mutant relative to the wild-type strain when grown under either activating condition. When cell integrity stress is prevented in glucose medium, however, glycogen levels are identical in the wild type and *psk1 psk2* mutant strain. An alternative explanation is that these two stimuli (glucose deprivation or cell integrity stress) initiate a stress response activating PAS kinase to increase partitioning of resources toward cellular stability.

Interestingly, Psk1 and Psk2 appear to have differential roles in response to the metabolic and cell integrity stimuli. Psk2 was transcriptionally downregulated by nonfermentative carbon source and was, therefore, unable to phosphorylate Ugp1 under this condition, whereas both Psk1 and Psk2 were activated by cell wall stress. Although Psk1 and Psk2 are transcriptionally regulated, both proteins were activated by either stimulus when expressed from a constitutive promoter. This implies that activation of PAS kinase by metabolic status or cell integrity stress occurs post-translationally. While the details of the activating mechanisms await future studies, we hypothesize that both pathways act through global conformational changes of PAS kinase. We have shown that the PAS domain of PAS kinase binds to and inhibits the kinase domain. It has also been proposed that this inhibitory interaction might be disrupted by a cellular metabolite directly binding to the PAS domain and displacing the kinase domain. It is, therefore, likely that the activation of PAS kinase in response to the metabolic stimulus occurs via direct binding of metabolite to the PAS domain. Consistently, we have observed that a form of PAS kinase that lacks the N-terminus (including the PAS domain) is no longer activated by the metabolic or cell integrity stimuli (Figure 5C).

As mentioned above, a possible PAS kinase-activating stimulus is the dramatic increase in mitochondrial biogenesis and metabolism observed in glucose derepression. It is of interest to note that mammalian PAS kinase is also activated under conditions of increased mitochondrial metabolism in cultured pancreatic β-cells (da Silva Xavier *et al*, 2004). The mechanisms for PAS kinase activation are still unclear in both yeast and mammals, but we hypothesize that this represents a conserved metabolic sensing function of PAS kinase. Thus, identification of the factors leading to PAS kinase activation in yeast may aid in understanding β-cell glucose sensing and the role of PAS kinase in diabetes and obesity.

Materials and methods

Yeast cells, culture media and materials

The W303-1a-derived *S. cerevisiae* strain JRY245 (MATa *his3-11,15 met15Δ0 leu2-3,112 ura3-52 lys2Δ25 trp1-1*) (Thomas and Rothstein, 1989) was obtained from David Stillman. Strains JRY276 (*psk1*::*HIS3 PSK2*), JRY 277 (*PSK1 psk2*::*KanMX4*) and JRY278 (*psk1*::*HIS3 psk2*::*KanMX4*) are derivatives of JRY245 made by homologous recombination. JRY506 (*reg1*::*HphMX4*), JRY514 (*reg1*::*HphMX4 snf1*::*URA3*), JRY853 (*psk2*::*KanMX4*), JRY854 (*rom2*::*HphMX4*) and JRY858 (*psk2*::*KanMX4 rom2*::*HphMX4*) are derivatives of JRY245 generated by homologous recombination. Integrity of recombination was verified by PCR. Tap-tagged strains JRY405 (MATα *PSK1-TAP:KanMX4 SUC2 mal mel gal2 CUP1 flo1 flo8-1*) and JRY406 (MATα *PSK2-TAP:KanMX4 SUC2 mal mel gal2 CUP1 flo1 flo8-1*) were obtained from Steve McKnight. Constitutive promoter strains, which are derivatives of JRY405 and JRY406 are as follows: JRY668 (*trp1Δ ura3Δ0 psk2*::*TRP1-pCYC1Δ-PSK2-TAP:KanMX4*), JRY670 (*trp1Δ ura3Δ0 psk2*::*TRP1-pCMD1-PSK2-TAP:KanMX4*), JRY672 (*trp1Δ ura3Δ0 psk2*::*TRP1-pSTE20-PSK2-TAP:KanMX4*), JRY680 (*trp1Δ ura3Δ0 psk1*::*TRP1-pCYC1Δ-PSK1-TAP:KanMX4*), JRY682 (*trp1Δ ura3Δ0 psk1*::*TRP1-pCMD1-PSK1-TAP:KanMX4*), JRY684 (*trp1Δ ura3Δ0 psk1*::*TRP1-pSTE20-PSK1-TAP:KanMX4*) and JRY674 (*trp1Δ ura3Δ0 psk2*::*TRP1-pSTE20-PSK2ΔN-TAP:KanMX4)*. The cell wall integrity pathway strains *wsc1*::*KanMX4*, *wsc2*::*KanMX4*, *wsc3*::*KanMX4*, *mpk1*::*KanMX4*, *mid2*::*KanMX4*, *mtl1*::*KanMX4*, *msb1*::*KanMX4*, *lre1*::*KanMX4*, *rom2*::*KanMX4* and *zds1*::*KanMX4*, *tus1*::*KanMX4* are derivatives of strain BY4742 MATa *his3D1 leu2D0 lys2D0 ura3D0* and were produced by the Saccharomyces Genome Deletion Project. The CYC1Δ promoter includes the sequence from −150 to −1 relative to

the translational start codon; CMD1 includes −400 to −15; and STE20 includes −428 to −2. All medium was prepared as described previously (Sherman, 1991), and cultures were maintained at 30°C unless otherwise stated. Cultures were harvested in log phase ($OD_{600} = 0.6–0.8$) for all analyses described.

Ion-exchange chromatography

Cell-free extracts for ion-exchange chromatography were prepared by resuspending 50 ml of log-phase cells in 10 ml XWA (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, pH 7.4) with 50 mM NaCl, 1:300 Protease Inhibitor Cocktail (Sigma) and 1:300 Phosphatase Inhibitor Cocktail 1 (Sigma). A 4 g portion of glass beads was added and the cells were intermittently vortexed for a total of 3 min. The resulting lysate was centrifuged at 20 000 *g* for 20 min, filtered through a 0.2 μm filter and loaded onto an MonoQ anion exchange column for fractionation as described previously (Smith and Rutter, 2007).

Immunoprecipitation of TAP-tagged PAS kinase

Cell-free extracts for immunoprecipitation were prepared by resuspending 50 ml of log-phase cells in 3 ml lysis buffer (20 mM HEPES pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, 50 mM NaF, 50 mM β-glycero-phosphate, 1:300 Protease Inhibitor Cocktail (Sigma) and 10% glycerol) and harvesting as described for chromatography. TAP-tagged PAS kinase was immunoprecipitated by incubating extract with 50 μl of IgG-Sepharose (Amersham Biosciences) at 4°C for 2 h. The beads were then washed four times with lysis buffer and split in half (for kinase assay and western blot analysis). The TAP-tagged PAS kinase was visualized by western blot analysis using antibody to detect the TAP tag (peroxidase anti-peroxidase antibody, Sigma P-1291).

In vitro phosphorylation of Ugp1

The purified PAS kinase protein was assayed for *in vitro* phosphorylation of Ugp1 using ^{32}P-ATP as described previously (Rutter *et al*, 2002) in 30 μl of reaction buffer containing 1 × kinase buffer, 2 mM ^{32}P-ATP, 5 μg Ugp1.

Quantification of phosphorylated Ugp1

For quantification of the *in vivo* assay of Ugp1-phosphorylation, the activity of Ugp1 in each fraction from the MonoQ column was assayed as described previously (Smith and Rutter, 2007). The percentage of phosphorylated Ugp1 was calculated by fitting the sum of two Gaussians to these data and determining the area under each peak (phosphorylated and unphosphorylated Ugp1) using Kaleidagraph (Synergy Software). For the *in vitro* PAS kinase assay, ^{32}P-Ugp1 was quantified using a Molecular Dynamics PhosphorImager system.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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