Saccharomyces cerevisiae cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase

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The *Saccharomyces cerevisiae* protein kinase Rim15p was identified previously as a stimulator of meiotic gene expression. Here, we show that loss of Rim15p causes an additional pleiotropic phenotype in cells grown to stationary phase on rich medium; this phenotype includes defects in trehalose and glycogen accumulation, in transcriptional derepression of *HSP12*, *HSP26*, and *SSA3*, in induction of thermotolerance and starvation resistance, and in proper G₁ arrest. These phenotypes are commonly associated with hyperactivity of the Ras/cAMP pathway. Tests of epistasis suggest that Rim15p may act in this pathway downstream of the cAMP-dependent protein kinase (cAPK). Accordingly, deletion of *RIM15* suppresses the growth defect of a temperature-sensitive adenylate-cyclase mutant and, most importantly, renders cells independent of cAPK activity. Conversely, overexpression of *RIM15* suppresses phenotypes associated with a mutation in the regulatory subunit of cAPK, exacerbates the growth defect of strains compromised for cAPK activity, and partially induces a starvation response in logarithmically growing wild-type cells. Biochemical analyses reveal that cAPK-mediated *in vitro* phosphorylation of Rim15p strongly inhibits its kinase activity. Taken together, these results place Rim15p immediately downstream and under negative control of cAPK and define a positive regulatory role of Rim15p for entry into both meiosis and stationary phase.

[Key Words: Rim15p; protein kinase A; yeast; Ras; cAMP; nutrient signaling]

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The cAMP-dependent protein kinase (cAPK) pathway in the yeast *Saccharomyces cerevisiae* is required for proper regulation of growth, cell cycle progression, and development in response to nutritional conditions. Cells deficient in cAPK activity stop growth, arrest in G₁, and show physiological changes normally associated with nutrient deprivation; these changes include the accumulation of trehalose and glycogen, enhanced expression of various genes (e.g., *SSA3*, *HSP12*, *HSP26*, *CTT1*, *UBI4*, and *ADH2*), and increased resistance to heat stress. In contrast, cells carrying mutations that yield elevated cAPK activity fail to arrest in G₁, are defective for trehalose and glycogen accumulation, rapidly lose viability, and remain highly sensitive to heat stress upon nutrient starvation (for review, see Tatchell 1986; Thevelein 1994). On the basis of these results, it was suggested that the central role of the yeast cAPK pathway is in signaling the nutrient status, thereby participating in the cell's decision to enter a quiescent state in G₁ that is equivalent to the G₀ state of higher eukaryotes. Accordingly, low levels of cAPK activity promote exit from the mi-

totic cell cycle and entry into G₀, while high levels of cAPK activity preclude access to G₀.

The activity of *S. cerevisiae* cAPK is regulated by a complex signaling pathway that includes two yeast homologs of mammalian *ras* proteins. The yeast *RAS1* and *RAS2* gene products (Ras) are small GTP-binding proteins that are activated by a GTP-exchange factor (GEF; encoded by *CDC25*) and inactivated by stimulation of the intrinsic GTPase-activity via GTPase-activating proteins (GAP; encoded by *IRA1* and *IRA2*). Activated GTP-bound Ras stimulates adenylate cyclase (encoded by *CYR1/CDC35*) to yield increased levels of cAMP that can be degraded by the low- and high-affinity phosphodiesterases encoded by the *PDE1* and *PDE2* genes, respectively. Binding of cAMP to the regulatory subunits of cAPK (encoded by *BCY1*) results in their dissociation from the catalytic subunits (encoded by three functionally redundant genes, *TPK1*, *TPK2*, and *TPK3*) and in stimulation of cAPK activity (for review and further details on the Ras/cAMP pathway, see Tatchell 1986; Gibbs and Marshall 1989; Broach and Deschenes 1990; Thevelein 1994). While the components of the Ras/cAMP pathway required for activation of cAPK are well established, little is known about the mechanisms of

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activation of the pathway by biological signals (i.e., nutrients) or about the potential biochemical targets of cAPK. These include enzymes involved in carbohydrate and phospholipid metabolism and various regulators of transcription, as well as proteins involved in synthesis and degradation of cAMP. Many of these potential targets, however, have not been unequivocally shown to be directly phosphorylated by cAPK, and it is likely that cAPK impinges on some of these targets rather indirectly (for review, see Broach and Deschenes 1990).

A fairly well established example of direct regulation by cAPK-catalyzed phosphorylation, by means of genetic, physiological, and biochemical analyses, is provided by the trehalose degrading enzyme trehalase (for review, see Thevelein 1996). Accumulation of the nonreducing disaccharide trehalose is an element of the adaptive response of yeast cells to nutrient starvation (Lillie and Pringle 1980). In general, resumption and stimulation of growth upon readdition of nutrients to starved cells are associated with mobilization of trehalose by rapid cAPK-dependent activation of neutral trehalase. Interestingly, it was suggested that not only the neutral trehalase but also the second key enzyme of trehalose metabolism, namely the trehalose-6-phosphate (Tre6P) synthase, may be regulated by cAPK-catalyzed phosphorylation (Panek et al. 1987). Accordingly, trehalose levels may be finely tuned in response to the nutritional status by reciprocal regulation (i.e., activation of neutral trehalase and inactivation of Tre6P synthase) via cAPK-mediated phosphorylation of the key enzymes of trehalose metabolism. However, although control of neutral trehalase by cAPK-dependent phosphorylation seems well established, there has been controversy as to whether Tre6P synthase is also regulated by cAPK-catalyzed phosphorylation (Vandercammen et al. 1989).

Tre6P synthase itself is part of a multimeric protein complex that is composed of at least four different subunits encoded by the genes *TPS1*, *TPS2*, *TPS3*, and *TSL1*. Recent studies indicated that Tps1p and Tps2p carry the catalytic activities of Tre6P synthase and Tre6P phosphatase, respectively, whereas Tps3p and Tsl1p were suggested to have regulatory and/or structural functions (Bell et al. 1992; De Virgilio et al. 1993; Vuorio et al. 1993; Reinders et al. 1997). A particularly surprising aspect of studies of the Tre6P synthase/phosphatase complex in *S. cerevisiae* was the finding that *tps1* mutants (including various allelic mutants) are defective not only for Tre6P synthesis but also for growth on glucose, apparently because of an uncontrolled influx of glucose into the glycolytic pathway. Therefore, it has been suggested that Tps1p may be involved in the control of glycolysis (for review, see Thevelein and Hohmann 1995; and references therein).

In view of this newly discovered role of Tps1p in regulation of the glycolytic pathway and its own possible regulation via the Ras/cAMP signaling pathway, identification of proteins that can interact with Tps1p may reveal novel regulatory mechanisms of both glucose influx and/or signal transduction via the Ras/cAMP pathway. To isolate such potential regulatory proteins, we

undertook a two-hybrid screen for proteins that interact with Tps1p. Here, we describe the identification of the Rim15p protein kinase, previously identified as a stimulator of meiotic gene expression (Vidan and Mitchell 1997), as a Tps1p-interacting protein. Deletion of *RIM15* results in a defective response of mutant cells to nutrient limitation, including a defect in trehalose accumulation, that is reminiscent of the effects caused by mutations that constitutively activate cAPK (e.g., *bcy1* and *RAS2^{Val19}*). Tests of epistasis as well as biochemical studies suggest that Rim15p acts immediately downstream of cAPK to control a broad range of physiological adaptations necessary for proper entry into stationary phase.

Results

Cloning and sequence analysis of RIM15

To identify previously uncharacterized genes whose products interact with the Tre6P synthase (Tps1p) of *S. cerevisiae*, we performed a two-hybrid screen (Fields and Sternglanz 1994; Zervos et al. 1993; see Materials and Methods). We rescued library plasmids from cells (EGY48; Table 1) in which both reporter genes showed galactose-dependent transcription and assigned the plasmids to three different classes by restriction mapping. Partial sequencing of plasmids of one class showed them to contain a previously unidentified gene with high homology to protein kinases. We decided to first study this gene and named it *TAK1* (for Tps1p-associated protein kinase). During the course of our studies, however, the same gene was identified as *RIM15* in an independent screen for mutations that cause reduced expression of *IME2* (Vidan and Mitchell 1997). In addition, the *TAK1/RIM15* sequence is identical to the open reading frame YFL033C identified as part of the Yeast Genome Sequencing Project (Murakami et al. 1995). We will refer to this gene and its product as *RIM15* and Rim15p, respectively. In accordance with Vidan and Mitchell (1997), we found *RIM15* to specify a 1770-residue polypeptide comprising a domain with high homology to serine/threonine protein kinases of the protein kinase C and protein kinase A subfamilies (see also Hunter and Plowman 1997). A notable feature of the predicted Rim15p amino acid sequence is the presence of five consensus sites for cAPK phosphorylation, Arg Arg X Ser (Edelman et al. 1987).

Analysis of the nine *RIM15* library plasmids isolated in the two-hybrid screen revealed that they all contained the same fragment of *RIM15*, corresponding to amino acids 761–1051 of the predicted full-length gene product. As shown in Table 2, this Rim15p_{761–1051}-activation domain fusion (AD) interacted with the Tps1p-DNA-binding domain fusion (DBD), but not with Tps2p-DBD, Tps3p-DBD, or Tsl1p-DBD. Because all four predicted proteins, Tps1p, Tps2p, Tps3p, and Tsl1p, share high homology over the entire length of the Tps1p sequence, these two-hybrid data indicate that the observed interaction between Rim15p_{761–1051}-AD and Tps1p-DBD is highly specific.

Table 1. List of yeast strains

Strain	Genotype	Source
YEF473	<i>MATa/α</i> <i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3</i>	Bi and Pringle (1996)
AR1	<i>MATa/α</i> <i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rim15Δ::kanMX2/RIM15</i>	this study
AR1-1B	<i>MATα</i> <i>his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2</i>	segregant from AR1
AR1-1C	<i>MATa</i> <i>his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2</i>	segregant from AR1
AR2	<i>MATa/α</i> <i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rim15Δ::kanMX2/rim15Δ::kanMX2</i>	AR1-1B X AR1-1C
SP1	<i>MATa</i> <i>ade8 his3 leu2 trp1 ura3</i>	Toda et al. (1985)
T16-11A	<i>MATa</i> <i>his3 leu2 trp1 ura3 bcy1-1</i>	Toda et al. (1985)
PD6517	<i>MATα</i> <i>ade8 leu2 trp1 cdc35-10</i>	Becher dos Passos et al. (1992)
NB11	<i>MATα</i> <i>ade8 leu2 trp1 cdc35-10 rim15Δ::kanMX2</i>	this study
S7-7A × S7-5A	<i>MATa/α</i> <i>ade8/ade8 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 TPK1/tpk1::URA3 TPK2/tpk2::HIS3 TPK3/tpk3::TRP1</i>	Toda et al. (1987)
NB13	<i>MATa/α</i> <i>ade8/ade8 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 TPK1/tpk1::URA3 TPK2/tpk2::HIS3 TPK3/tpk3::TRP1 RIM15/rim15Δ::kanMX2</i>	this study
NB13-1D	<i>MATα</i> <i>ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1 rim15Δ::kanMX2</i>	segregant from NB13
NB13-14D	<i>MATa</i> <i>ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 rim15Δ::kanMX2</i>	segregant from NB13
EGY48	<i>MATα</i> <i>his3 trp1 ura3 LEU2::pLexAop6-LEU2</i>	Zervos et al. (1993)

RIM15 is required for proper entry into stationary phase

To determine the consequences of the loss of Rim15p, we replaced the complete *RIM15* coding region by a PCR-based gene deletion method using the *kanMX2* module (Wach et al. 1994; see Materials and Methods). The heterozygous *RIM15/rim15Δ* diploid AR1 was sporulated and the deletion was shown to segregate 2:2 as judged by geneticin resistance of the resulting colonies. Two tetrads were checked by PCR and Southern blotting, and, in each case, the presence of the selectable marker correlated with the shift in fragment size expected for the deleted alleles (data not shown). Thus, *RIM15* is not essential for growth or germination.

For further analysis of the effects of *rim15Δ* we constructed a homozygous diploid *rim15Δ/rim15Δ* strain (AR2) by mating an appropriate pair of geneticin-resistant segregants of AR1 (Table 1). The *rim15Δ/rim15Δ* strain had no obvious growth defect at 30°C or 37°C on all carbon sources we tested, including glucose, fructose, sucrose, raffinose, maltose, glycerol, and ethanol (data not shown). Because our two-hybrid analyses suggested that Rim15p may interact with Tps1p, we assayed the *rim15Δ/rim15Δ* strain for its capacity to accumulate trehalose during a mild heat shock and in stationary phase. Although the *rim15Δ/rim15Δ* mutant accumulated wild-type levels of trehalose during a 1-hr heat shock at 42°C (i.e., 0.345 ± 0.033 and 0.377 ± 0.009 gram/gram protein for the *rim15Δ/rim15Δ* mutant and the isogenic wild type, respectively), it was found to contain very low amounts of trehalose in stationary phase when compared with the isogenic wild type (Table 3). Surprisingly, this deficiency in stationary phase-induced trehalose accumulation was not reflected in any detectable changes in

the activities of the two key enzymes of trehalose metabolism, namely Tre6P synthase and neutral trehalase.

As the *rim15Δ/rim15Δ* mutant was found to be defective for trehalose synthesis upon entry into stationary phase, we also determined a variety of other phenotypic traits characteristic of stationary phase cells (Werner-Washburne et al. 1993). The *rim15Δ/rim15Δ* mutant was found to be normal for derepression of invertase (Table 3). However, it was impaired in its ability to accumulate glycogen, to induce *SSA3* transcription (as measured by induction of an *SSA3-lacZ* fusion gene), to acquire thermotolerance, and to arrest properly in G₁ (as measured by the percentage of budded cells) upon entry into stationary phase (Table 3). As a result, the strain was also highly sensitive to prolonged starvation (e.g., 10 days in

Table 2. Two-hybrid interactions between Rim15p and the subunits of the trehalose-6-phosphate synthase complex in *Saccharomyces cerevisiae*

AD fusion	DBD fusions			
	<i>TPS1</i>	<i>TPS2</i>	<i>TPS3</i>	<i>TSL1</i>
<i>RIM15-P</i>	46.8 ± 12.4	6.0 ± 4.0	1.3 ± 0.6	2.4 ± 1.0
pJG4-5	2.0 ± 1.4	1.1 ± 0.3	3.1 ± 2.5	2.3 ± 1.4

Possible interactions between Rim15p and Tps1p, Tps2p, Tps3p, or Tsl1p were detected using the two-hybrid system as described in Materials and Methods. Numbers represent mean β-galactosidase activities (in Miller units) ± S.D.s from three independent transformants for each pair of plasmids. pJG4-5 indicates the AD vector without insert. Note that the Tps1p-DBD, Tps2p-DBD, Tps3p-DBD, and Tsl1p-DBD fusions were all shown previously to interact with a Tps1p-AD fusion (Reinders et al. 1997). *RIM15-P* codes for an internal part of Rim15p comprising a section of the kinase domain (amino acids 761–1051).

Table 3. Effects of RIM15 deletion

	<i>RIM15/RIM15</i> ^a		<i>rim15Δ/rim15Δ</i> ^a	
	log	stat	log	stat
Enzymes and metabolites				
Tre6P synthase (μmole/sec per gram protein)	0.33	1.32	0.37	1.17
trehalase (μmole/sec per gram protein)	0.09	0.17	0.05	0.16
invertase (μmole/sec per gram protein)	2.00	12.93	1.93	12.06
<i>SSA3-lacZ</i> induction ^b (Miller units)	4.30	105.20	2.40	16.30
trehalose (gram/gram protein)	<0.001	0.199	<0.001	0.009
glycogen (mg/gram protein)	1.63	38.53	0.93	11.36
Thermotolerance ^c (% survival)	0.13	35.60	0.07	0.03
Stationary phase survival ^d (%)		100.00		20.20
Budded cells ^e (%)	65.70	0.90	65.70	24.30

^aWild-type and *rim15Δ/rim15Δ* strains were YEF473 and AR2, respectively. All experiments were carried out on YPD (2% glucose) medium using either log phase (log) or 4-day-old stationary phase (stat) cells, except where otherwise stated. Values represent means of at least three independent experiments; S.D.s were, in each case, <10% of the corresponding means.

^bβ-Galactosidase activities were measured to monitor the induction of an *SSA3-lacZ* fusion gene (from plasmid pWB204Δ-236).

^cThermotolerance was measured as the percent survival following a heat shock for 8 min at 50°C (log-phase cells) or 20 min at 53°C (stationary-phase cells).

^dThe percentage of viable cells was determined by the colony-forming efficiency on YPD agar of 10-day-old stationary-phase cultures.

^eThe percentage of budded cells was determined by microscopic examination of at least 200 cells.

stationary phase). Finally, the *rim15Δ/rim15Δ* mutant also exhibited much lower sporulation efficiency ($0.6\% \pm 0.5$) than the wild type ($31.1\% \pm 5.6$). Thus, the *RIM15* gene is required for sporulation and for proper entry into stationary phase.

The observed pleiotropic response to nutrient limitation and the defect in sporulation caused by deletion of *RIM15* are reminiscent of the effects of two previously described mutations in the Ras/cAMP pathway, namely *RAS2^{Val19}* and *bcy1*, which both result in uncontrolled activation of cAPK (Kataoka et al. 1984; Cannon and Tatchell 1987; Toda et al. 1987). Thus, Rim15p might be involved in signaling the status of nutrient supply (or limitation) at some point in the Ras/cAMP pathway. Therefore, we analyzed the transcription patterns in wild-type and *rim15Δ/rim15Δ* cells of various genes (including *SSA3*, *HSP12*, *UBI4*, and *ADH2*) that are known to be under negative control of cAPK activity (Tanaka et al. 1988; Cherry et al. 1989; Boorstein and Craig 1990; Varela et al. 1995). A comparison of transcript levels during exponential phase, diauxic shift, post-diauxic shift, and stationary phase in wild-type and *rim15Δ/rim15Δ* cells is shown in Figure 1. Although the wild-type strain showed the expected pattern of transcriptional repression during exponential growth and transcriptional derepression after the diauxic shift for all five of these genes, the *rim15Δ/rim15Δ* strain was seriously defective for transcriptional derepression after the diauxic shift of *SSA3*, *HSP12*, and *HSP26*, but not for derepression of *UBI4* and *ADH2*. In fact, derepression of *ADH2*, the only gene of this group whose expression is known to be controlled by cAPK-dependent inactivation of the transcriptional activator Adr1p (Cherry et al. 1989), was even found to be enhanced during the post-diauxic phase and in stationary phase. We also examined the expression pattern of the cold-inducible *SSB1* gene, a member of the Hsp70p subfamily, whose transcription is repressed

upon entry into stationary phase (Werner-Washburne et al. 1989). No significant difference in the *SSB1* repression pattern in wild-type and *rim15Δ/rim15Δ* cells during and after the diauxic shift was found (Fig. 1). Taken together, these results indicate that Rim15p is required for the stationary phase-induced transcriptional derepression (or activation) of a subset of genes (*SSA3*, *HSP12*, and *HSP26*) known to be negatively controlled by cAPK. In accordance with such a role of Rim15p, expression of *RIM15* itself was found to be very weak during exponential growth but to be highly induced during the diauxic shift and the subsequent post-diauxic and stationary phases (Fig. 1).

Figure 1. Abundance of various mRNA species as wild-type (YEF473) and *rim15Δ/rim15Δ* (AR2) mutant cells grow to stationary phase in YPD medium. Total RNAs extracted from cells in exponential phase (0.5 day), diauxic shift phase (1 day), post-diauxic shift phase (2 and 3 days), and stationary phase (4–8 days) were extracted at the times indicated and equal amounts (10 μg) were probed with *RIM15*, *SSA3*, *HSP12*, *HSP26*, *UBI4*, *SSB1*, and *ADH2* fragments after electrophoresis and blotting. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining.

Loss of Rim15p suppresses *cdc35^{ts}* and total loss of cAPK

To elucidate whether Rim15p may act in the Ras/cAMP pathway, we determined whether the loss of Rim15p could suppress the conditional growth of a temperature-sensitive adenylate cyclase mutant. To this end, the *rim15::kanMX2* deletion was introduced into strain PD6517 (*cdc35-10*), and three geneticin-resistant colonies were tested for growth at the non-permissive temperature of 35°C. In all cases, deletion of *RIM15* was found to allow growth at this temperature. Thus, *cdc35-10* (PD6517) and *cdc35-10 rim15Δ* (NB11) cultures had an OD₆₀₀ of 0.15 ± 0.06 and 4.47 ± 0.26 , respectively, after inoculation (OD₆₀₀ 0.05) and growth for 2 days at 35°C in liquid YPD (2% glucose) medium.

To examine whether Rim15p may function immediately downstream of Cdc35p or at a later step in the Ras/cAMP pathway, we tested whether deletion of *RIM15* could suppress the complete loss of cAPK. To this end, a heterozygous *TPK1/tpk1 TPK2/tpk2 TPK3/tpk3* diploid strain (S7-7A × S7-5A) was transformed to geneticin-resistance with the *rim15::kanMX2* deletion construct and allowed to sporulate, and the asci were dissected on YPD agar. As expected *tpk1 tpk2 tpk3 RIM15* spores failed to germinate (as judged by segregation of the auxotrophic markers). However, *tpk1 tpk2 tpk3 rim15Δ* (*tpk rim15Δ*) spores were viable. In a complementary experiment, a *tpk rim15Δ* strain was transformed either with a plasmid that allows galactose-inducible expression of *RIM15* (YCpIF2-*RIM15*) or with the corresponding control plasmid (YCpIF2). As expected, *tpk rim15Δ* cells containing the control plasmid were able to grow on both glucose- and galactose-containing medium. In contrast, *tpk rim15Δ* cells containing YCpIF2-*RIM15* were able to grow on glucose-containing medium but failed to grow on galactose-containing medium (Fig. 2). Together, these results show that the loss of Rim15p suppresses the lethal effect of total loss of cAPK.

Figure 2. Suppression of the *tpk* growth defect by *rim15Δ*. *tpk1 tpk2 tpk3 rim15Δ* cells (NB13-14D) were transformed with plasmids YCpIF2 (control) and YCpIF2-*RIM15* (allowing galactose-dependent expression of *RIM15*), streaked on SD media containing either glucose or galactose as carbon source, and incubated for 4 days at 30°C.

Figure 3. Northern blot analysis of gene expression in exponentially growing and stationary phase *TPK RIM15* (SP1), *TPK rim15Δ* (NB13-1D), and *tpk rim15Δ* (NB13-14D) cells. Total RNA was extracted from log phase (L) and stationary phase (S1, 2 days; S2, 4 days after glucose depletion) cells grown in YPD medium. For further details see Fig. 1, legend.

Because *tpk rim15Δ* cells were viable, we were able to compare *TPK rim15Δ* and *tpk rim15Δ* cells and to examine whether, as expected for a downstream effector, *rim15Δ* was epistatic over *tpk* mutations with respect to various stationary-phase-associated phenotypes. *SSA3*, *HSP12*, and *HSP26* were repressed in logarithmically growing wild-type cells and induced in stationary phase cells (Fig. 3, lanes 1–3). In *rim15Δ* mutants, these genes were found to be repressed both in logarithmically growing and in stationary phase cells independent of the presence or absence of a functional *TPK* gene (Fig. 3, lanes 4–9). As a control for the physiological status of the cells, *SSB1* was shown to be highly expressed in log phase cells and repressed in stationary phase cells of all three strains (Fig. 3, lanes 1–9). Like *TPK rim15Δ* cells, *tpk rim15Δ* cells were also defective for the accumulation of trehalose (0.027 ± 0.005 gram/gram protein vs. < 0.01 gram/gram protein for *TPK rim15Δ* cells). Thus, *rim15Δ* is largely epistatic over *tpk* mutations with respect to these hallmarks of stationary phase (i.e. transcriptional activation/derepression of *SSA3*, *HSP12*, and *HSP26* and trehalose accumulation). In contrast, *tpk rim15Δ* cells had reduced growth rates at 30°C (0.16 ± 0.01 /hr versus 0.27 ± 0.01 /hr for *TPK rim15Δ* cells), exhibited higher levels of thermotolerance ($6.3 \pm 1.6\%$ vs. $0.07 \pm 0.01\%$ survival after incubation for 20 min at 53°C for *TPK rim15Δ* cells) and higher rates of survival in stationary phase ($50.7 \pm 9.8\%$ vs. $4.6 \pm 0.8\%$ after 10 days in stationary phase for *TPK rim15Δ* cells), and hyperaccumulated glycogen (as measured by iodine staining) when compared with *TPK rim15Δ* cells. Thus, the *TPK* status still affected these phenotypes in *rim15Δ* cells. The significance of these results is discussed below.

RIM15 overexpression suppresses *bcy1-1*, exacerbates the growth defect of a strain partially compromised for cAPK activity, and partially mimics a nutrient limited state in wild-type cells

The data presented above suggest that Rim15p activity may be under direct or indirect negative control by cAPK and that Rim15p may be responsible for the induction of

Table 4. Suppression of *bcy1-1* phenotypes by overexpression of *RIM15*

Strain	Relevant genotype	Plasmid	Trehalose (gram/gram protein)	<i>SSA3-lacZ</i> induction (Miller units)	Thermotolerance (% survival)
SP1	<i>BCY1</i>	YCp <i>ADH1</i>	0.39 ± 0.05	137.8 ± 37.5	39.8 ± 3.7
SP1	<i>BCY1</i>	YCp <i>ADH1-RIM15</i>	0.56 ± 0.11	299.9 ± 76.0	73.0 ± 2.4
T16-11A	<i>bcy1-1</i>	YCp <i>ADH1</i>	0.12 ± 0.06	41.4 ± 11.1	12.6 ± 2.9
T16-11A	<i>bcy1-1</i>	YCp <i>ADH1-RIM15</i>	0.24 ± 0.02	123.9 ± 25.7	43.0 ± 10.9

Cells were grown to stationary phase (5 days) on SD medium containing 1% glucose. Values represent means ± S.D.s of three to six independent experiments. β -Galactosidase activities were measured to monitor the induction of an *SSA3-lacZ* fusion gene (from plasmid pWB204 Δ -236). Thermotolerance was measured as the survival following a heat shock for 20 min at 50°C.

a subset of the physiological changes triggered by nutrient limitation including the synthesis of trehalose and the transcriptional derepression (or activation) of *SSA3*, *HSP12*, and *HSP26*. If this model were correct, one would expect that overexpression of *RIM15* should, on the one hand, revert phenotypes associated with uncontrolled, constitutive activation of cAPK and, on the other hand, exacerbate phenotypes associated with attenuated cAPK activity. In accordance with these expectations, overexpression of *RIM15* under the control of the *ADH1* promoter (YCp*ADH1-RIM15*) partially suppressed the defect in trehalose accumulation and completely suppressed the defects in *SSA3-lacZ* induction (from pWB204 Δ -236) and thermotolerance acquisition of *bcy1-1* cells entering stationary phase (Table 4). In addition, overexpression of *RIM15* caused a significant increase of stationary-phase-induced trehalose accumulation, *SSA3-lacZ* induction, and thermotolerance acquisition in wild-type cells (Table 4; see also below). Furthermore, overexpression of *RIM15* was found to exacerbate the growth defect of a temperature-sensitive adenylate cyclase mutant (*cdc35-10*): Although this particular mutant grew well at 34°C and at 27°C when harboring the control plasmid, it was defective for growth at 34°C, but not at 27°C, when overexpressing *RIM15* (Fig. 4).

To study further the effects of *RIM15* overexpression in wild-type cells, strain YEF473 containing the reporter plasmid pWB204 Δ -236 was transformed with either YCpIF2-*RIM15* (allowing galactose-inducible expression of *RIM15*) or YCpIF2 (control) and analyzed for trehalose levels, *SSA3-lacZ* induction, and thermotolerance after growth for 16 hr (log phase) or 4 days (stationary phase) on galactose-containing medium. As shown in Table 5, overexpression of *RIM15* significantly enhanced the levels of trehalose, *SSA3-lacZ* induction, and thermotolerance in log as well as in stationary-phase cells when compared with the corresponding control. Thus, physiological adaptations associated with stationary phase are partially induced in log-phase cells and enhanced in stationary-phase cells by overexpression of *RIM15*.

Rim15p protein kinase activity is negatively regulated by cAPK-dependent phosphorylation

The epistasis analyses presented above suggest that Rim15p serves to inhibit cell growth and to promote

stationary phase entry and that its kinase activity may be attenuated by direct cAPK-dependent phosphorylation. This would mark Rim15p as the first protein kinase known to constitute a downstream effector of cAPK in *S. cerevisiae*. To test this model, we first analyzed whether Rim15p has protein kinase activity and whether it could phosphorylate itself in vitro. To this end, a functional GST-Rim15p fusion protein was expressed in wild-type *S. cerevisiae* cells, purified by glutathione affinity chromatography, and incubated with [γ -³²P]ATP. This procedure resulted in phosphorylation of a 223-kD polypeptide (expected size for GST-Rim15p) and of a variety of smaller polypeptides (Fig. 5A). Immunoblot analysis with anti-GST antibodies also revealed the presence of a 223-kD polypeptide as well as a similar array of smaller polypeptides that were not present in GST control extracts (data not shown). From these data, we infer that the 223-kD band corresponds to the GST-Rim15p and that the smaller bands represent proteolytic fragments of it. To eliminate the possibility that in vitro Rim15p phosphorylation is catalyzed by a protein kinase coprecipitating with GST-Rim15p and to assess the extent of autophosphorylation, we examined the autophosphorylation activity of an ATP-binding-deficient GST-Rim15p-K823Y fusion protein. This mutant fusion protein has lysine 823, corresponding to the conserved lysine shown to be required for ATP binding and kinase activity of known protein kinases (Saraste et al. 1990),

Figure 4. Overexpression of *RIM15* exacerbates the temperature-sensitive growth defect of a *cdc35-10* mutant. Strain PD6517 (*cdc35-10*) was transformed with plasmids YCp*ADH1* and YCp*ADH1-RIM15*, streaked on SD medium, and incubated for 3 days at the temperatures indicated.

Table 5. Effects of RIM15 overexpression

Strain	Relevant genotype	Trehalose (gram/gram protein)		SSA3-lacZ induction (Miller units)		Thermotolerance (% survival)	
		log	stat	log	stat	log	stat
YEF473	wild-type (YCpIF2)	0.006 ± 0.002	0.238 ± 0.030	12.6 ± 3.0	429.0 ± 61.9	0.9 ± 0.5	77.1 ± 8.5
YEF473	wild-type (YCpIF2-RIM15)	0.063 ± 0.008	0.364 ± 0.027	37.0 ± 3.7	589.0 ± 43.7	4.9 ± 1.6	98.5 ± 18.0

Cells were grown to log phase (log) or stationary phase (stat; 4 days) on SD medium containing 2% galactose and 1% raffinose to induce *GAL1*-driven transcription of *RIM15* in YCpIF2-*RIM15*. Values represent means ± S.D.s of three experiments. β -Galactosidase activities were measured to monitor the induction of an *SSA3-lacZ* fusion gene (from plasmid pWB204Δ-236). Thermotolerance was measured as the survival following a heat shock for 4 min (log-phase cells) or 20 min (stationary-phase cells) at 50°C.

replaced by a tyrosine. As shown in Figure 5A, autophosphorylation of GST-Rim15p-K823Y was reduced by about 90%. Thus, the major part of the Rim15p in vitro phosphorylation results from an autocatalytic reaction.

Then we examined the role of cAPK in Rim15p phosphorylation. Incubation of GST-Rim15p and GST-Rim15p-K823Y with bovine cAPK and [γ -³²P]ATP led to extensive phosphorylation of both fusion proteins (Fig. 5A; similar results were obtained using bacterially expressed GST-Tpk1p instead of bovine cAPK. Since we found the activity of GST-Tpk1p to be highly unstable, we used bovine cAPK in our experiments). The lower

phosphorylation level after cAPK-treatment of GST-Rim15p-K823Y compared with GST-Rim15p may be explained by the absence of autophosphorylation in the former fusion protein. Because Rim15p can be phosphorylated by cAPK, we addressed the significance of this event for Rim15p activity. For this assay, α -casein, which was found to be efficiently phosphorylated by Rim15p, was used as exogenous substrate. As shown in Figure 5B, the ability of Rim15p to phosphorylate α -casein was significantly reduced (>75%) by preincubation with bovine cAPK. This cAPK-mediated reduction could be completely prevented if cAPK inhibitor was present

Figure 5. cAPK-dependent phosphorylation of Rim15p inhibits its kinase activity. (A) GST-Rim15p, GST-Rim15p-K823Y, and GST proteins were purified and analyzed for autophosphorylation activity and their potential to be phosphorylated by cAPK. Accordingly, equal amounts of the fusion proteins (verified by immunoblotting with anti-GST antibodies) were incubated with [γ -³²P]ATP either in the absence (-cAPK) or in the presence (+cAPK) of cAPK as described in Materials and Methods. Phosphorylation levels were quantitated by PhosphorImager analysis and expressed as percent of the GST-Rim15p-phosphorylation level (+cAPK). (B) To analyze the effect of cAPK-dependent phosphorylation of Rim15p, equal amounts of GST-Rim15p, GST-Rim15p-K823Y, and GST (verified by immunoblotting as in A) were preincubated with unlabeled ATP and either no further additions, with cAPK, with cAPK and cAPK inhibitor, or with cAPK inhibitor alone, as indicated. The samples were washed extensively and assayed for α -casein phosphorylation in the presence of cAPK inhibitor and [γ -³²P]ATP. The levels of α -casein phosphorylation were quantitated by PhosphorImager analysis and expressed as percent of the control (level of α -casein phosphorylation after preincubation of GST-Rim15p in the absence of both cAPK and cAPK inhibitor). (C) Equal amounts of GST-Rim15p and GST-Rim15p-S709A/S1094A/S1416A/S1463A/S1661A [GST-Rim15p-5x(RRXS/A)] were analyzed as in B.

during the preincubation. In control experiments with GST or GST-Rim15p-K823Y we found no detectable or only residual (<4%) phosphorylation of α -casein, respectively. In accordance with these results, the corresponding *GST* and *GST-rim15^{K823Y}* genes, but not the *GST-RIM15* gene, failed to complement the *rim15 Δ* mutation (data not shown).

As mentioned above, the predicted Rim15p amino acid sequence contains five Arg Arg X Ser consensus sites for cAPK phosphorylation (Edelman et al. 1987). If Rim15p kinase activity is down-regulated by cAPK-dependent phosphorylation, replacement of Ser with Ala at these five consensus sites should yield a cAPK-unresponsive Rim15p mutant protein. As expected, we found that a GST-Rim15p-S709A/S1094A/S1416A/S1463A/S1661A quintuple mutant protein [GST-Rim15p-5x(RRXS/A)] could not be inhibited in vitro by preincubation with cAPK (Fig. 5C). Taken together, these data show that cAPK-mediated phosphorylation of Rim15p inhibits its kinase activity.

Discussion

We identified the Rim15p protein kinase in a two-hybrid screen for potential interactors with Tps1p, the small subunit of the Tre6P synthase. Loss of Rim15p was found to cause a highly pleiotropic phenotype in cells entering stationary phase, including a defect in trehalose synthesis, which suggests a role for Rim15p in nutrient signal transduction. Our results are most simply interpreted in a model in which Rim15p functions in the Ras/cAMP pathway downstream of cAPK to regulate a set of physiological adaptations necessary for proper entry into stationary phase. Several observations support such a model. First, deletion of *RIM15* results in a defective response to nutrient limitation of mutant cells entering stationary phase that is reminiscent of the effects caused by mutations that constitutively activate cAPK (e.g., *bcy1* and *RAS2^{Val19}*). Accordingly, *rim15 Δ* cells are seriously defective in trehalose accumulation, in transcriptional derepression of *SSA3*, *HSP12*, and *HSP26*, in induction of thermotolerance and starvation resistance, as well as in proper G₁ arrest when entering stationary phase. Second, deletion of *RIM15* suppresses the growth defect at elevated temperatures of a temperature-sensitive adenylate cyclase mutant (*cdc35-10*) and allows growth of cells that lack all three cAPK-encoding genes. Third, overexpression of *RIM15* suppresses the defects in trehalose accumulation, transcriptional derepression of *SSA3*, and thermotolerance acquisition in stationary cells of a *bcy1-1* mutant, exacerbates the growth defect of a strain compromised for cAPK activity (*cdc35-10*), and partially induces a starvation response in logarithmically growing wild-type cells. Fourth, cAPK-mediated phosphorylation of Rim15p, but not of a GST-Rim15p-5x(RRXS/A) quintuple mutant protein with Ser-to-Ala mutations at the consensus sites for cAPK phosphorylation, results in a strong reduction of the Rim15p protein kinase activity in vitro.

Despite the broad physiological overlap of the effects

of cAPK hyperactivity and of loss of Rim15p, there are some notable differences. In contrast to *rim15 Δ* , mutations that cause constitutive activation of cAPK such as *bcy1* or *RAS2^{Val19}* are generally associated with constitutive activation of neutral trehalase, complete inability to accumulate glycogen, and gluconeogenic growth defects (Kataoka et al. 1984; Toda et al. 1987; Cameron et al. 1988). A model that may account for these differences is that cAPK constitutes a point of divergence in the Ras/cAMP pathway and that Rim15p acts beyond this specific point (Fig. 6). Accordingly, cAPK may on the one hand transmit the signal of the Ras/cAMP pathway to Rim15p and on the other hand directly control several other cellular mechanisms such as the activation of neutral trehalase (for review, see Thevelein 1996), the inhibition of glycogen synthesis (Hardy et al. 1994 and references therein), and the inhibition of gluconeogenic growth (Müller and Holzer 1981; Mazón et al. 1982; François et al. 1984; Cherry et al. 1989). If this model were correct, *rim15 Δ* should be epistatic to *tpk* mutations with respect to some but not all phenotypes. Our results support such a conclusion. Accordingly, we found that a *tpk rim15 Δ* strain, like a *TPK rim15 Δ* strain, was defective for derepression of *SSA3*, *HSP12*, and *HSP26* as well as for trehalose accumulation upon entry into stationary phase, suggesting that these processes are downstream of and controlled by Rim15p. In contrast, the *tpk rim15 Δ* strain was still found to exhibit a reduced

Figure 6. Model for the cAMP-dependent protein kinase (cAPK)-Rim15p pathway. Glucose-repression as well as post-translational inhibition via cAPK-dependent phosphorylation, and potentially an additional yet-to-be-identified mechanism, result in low Rim15p kinase activity in log phase. During the postdiauxic shift, glucose derepression of *RIM15* (illustrated by the increase in size of the Rim15p protein), down-regulation of cAPK-dependent inhibition, and potential activation by yet-to-be-identified mechanisms result in high Rim15p kinase activity. Rim15p acts as a positive regulator of stationary phase entry and, consequently, as a negative regulator of growth. (Arrow) Positive interaction; (bar) negative interaction. Bold arrows and bars denote high activity. Dashed arrows and bars refer to potential interactions. Accumulation of trehalose and glycogen is indicated with bold letters. Inactive proteins are on white and active proteins are on shaded backgrounds. Nth1p, Tps1p, and Tps2p denote neutral trehalase, Tre6P synthase, and Tre6P phosphatase, respectively. For further details, see text.

growth rate, to acquire higher levels of thermotolerance, to hyperaccumulate glycogen, and—possibly as a result of the higher glycogen content—to have significantly enhanced levels of stationary phase survival when compared to the *TPK rim15Δ* strain. Thus, control over these processes may be exerted to some extent at the level of cAPK and therefore be at least partially independent of Rim15p. In this context, the seemingly contradictory finding that stationary *TPK rim15Δ* mutants had reduced levels of glycogen may be reconciled with our model if Rim15p would be involved in mechanisms of cAPK feedback inhibition. We are currently exploring this possibility in further detail.

Several protein kinases whose functions may partially overlap with the ones of the Ras/cAMP pathway have been described previously. One of these protein kinases is Snf1p, which functions primarily in glucose repression by its negative control of transcriptional factors such as Mig1p (for review, see Ronne 1995). Genetic studies indicated that not all Snf1p functions are mediated by Mig1p, though, and that Snf1p controls glycogen accumulation, acquisition of thermotolerance, and proper G₁ arrest upon depletion of external glucose in parallel and antagonistically to cAPK (Thompson-Jaeger et al. 1991; Woods et al. 1994; Huang et al. 1996). In this context, two other genes encoding protein kinases, namely *SCH9* and *YAK1*, have been isolated by genetic screens for growth-related effectors of cAPK (Toda et al. 1988; Garrett and Broach 1989). However, their corresponding gene products may both define separate nutrient signaling pathways that also act parallel to the Ras/cAMP pathway (Toda et al. 1988; Denis and Audino 1991; Garrett et al. 1991; Thompson-Jaeger et al. 1991; Hartley et al. 1994). Thus, even though the roles of Snf1, Sch9, and Yak1 in the Ras/cAMP pathway are not understood, the available evidence suggests that all three protein kinases may functionally overlap with and act in parallel to the Ras/cAMP pathway. Therefore, Rim15p appears to be the first protein kinase identified as constituting a downstream effector of cAPK in *S. cerevisiae*.

Consistent with both the proposed role of Rim15p as an activator of stationary phase entry and the observation that *rim15Δ* caused no obvious defect in exponentially, glucose-grown cells, the level of *RIM15* mRNA and Rim15p protein were found to be very low during growth on glucose and to increase significantly upon glucose exhaustion (Vidan and Mitchell 1997; this study). Because *RIM15* is transcriptionally repressed in the presence of glucose, it is not surprising that replacement of *RIM15* with the *rim15^{5x(RRXS/A)}* mutant gene did not cause any significant effects in exponentially growing cells (data not shown). However, we also found that the effects of *rim15^{5x(RRXS/A)}* overexpression did not significantly differ from the ones observed following *RIM15* overexpression (i.e., both genes caused a similar, partial induction of stationary phase characteristics in exponentially growing cells; Table 5 and data not shown). This observation may be explained if overexpression of *RIM15* results in Rim15p protein levels that exceed a certain threshold level beyond which Rim15p may es-

cape cAPK-dependent down-regulation. Accordingly, maximal induction of stationary phase characteristics in log phase cells would already be achieved by overexpression of the *RIM15* wild-type gene. The rather moderate level of induction of these stationary phase characteristics in log phase cells may be attributable to the presence of activated cAPK, which indirectly counteracts the effects of Rim15p (e.g., by activation of neutral trehalase), and/or to potential cAPK-independent mechanisms involved in the control of Rim15p kinase activity. The latter would raise the intriguing possibility that Rim15p—besides the observed regulation by glucose repression and cAPK-dependent inhibition—is subjected to down-regulation and/or activation by at least one other, yet unidentified cAMP-independent nutrient signaling pathway. In this context, it is worth noting that a previous report suggested the existence of cAMP-independent mechanisms for regulation of proper stationary phase entry including trehalose accumulation, glycogen synthesis, and the development of thermotolerance (Cameron et al. 1988). Therefore, future studies should address the question of whether any of the potentially separate nutrient signaling pathways may converge at and be integrated by Rim15p.

A further interesting aspect of our study is the possibility that the *TPS1*-encoded Tre6P synthase may be a direct target of Rim15p. This suggestion is supported by several observations. First, Rim15p shows specific two-hybrid interaction with Tps1p. Second, loss of Rim15p causes a defect in stationary phase-induced trehalose accumulation. Third, overexpression of *RIM15* not only significantly induces trehalose synthesis in log-phase cells but also enhances trehalose accumulation in stationary phase cells. Even though these data do not exclude the possibility that Rim15p activates Tre6P synthase rather indirectly, they would be consistent with a model in which Tps1p is positively regulated by Rim15p-dependent phosphorylation. As intriguing as this model may be, it is not easily reconciled with the observation that the in vitro-measured Tre6P synthase of stationary phase *rim15Δ/rim15Δ* cells was found to be fully active, even though no trehalose was formed in vivo. This inconsistency may be explained, however, if Tre6P synthase were artificially activated by limited proteolysis during the sampling procedure as has been suggested earlier (Londesborough and Vuorio 1991; Vicente-Soler et al. 1991). Taken together, it is clear that further detailed biochemical characterization of Tps1p is necessary to elucidate whether its activity may be regulated via phosphorylation/dephosphorylation and whether Rim15p is directly or rather indirectly involved in such a process. Because recent evidence suggested that Tps1p is also involved in the control of glucose flux into glycolysis, possibly through a specific mechanism independent of trehalose synthesis (for review, see Thevelein and Hohmann 1995), such analyses may not only contribute to our understanding of the regulation of trehalose synthesis but may also help defining the potential trehalose synthesis-independent function of the yeast Tps1p protein.

Recently, Rim15p was identified independently through a mutation that caused reduced expression of *IME2* (Vidan and Mitchell 1997). Current knowledge suggests that Rim15p may act to stimulate formation of the Ime1–Ume6 complex, which in turn acts as a transcriptional activator of early meiotic gene expression (Bowdish et al. 1995; Rubin-Bejerano et al. 1996; Vidan and Mitchell 1997). In view of both these recent findings on Rim15p and the long standing suggestion that entry into meiosis is at least partially regulated by starvation through the Ras/cAMP pathway (Matsumoto et al. 1983; Smith and Mitchell 1989; Matsuura et al. 1990), our present study, placing Rim15p immediately downstream of cAPK, may finally link the Ras/cAMP pathway to early meiotic gene expression.

Materials and methods

Strains, media, and microbiological and recombinant DNA methods

The *S. cerevisiae* strains used in this study are listed in Table 1. *Escherichia coli* strain JMB9 ($[r^{-}m^{-}]$ *trpF*) was used to rescue pJG4-5-based plasmids from strain EGY48 as described earlier (De Virgilio et al. 1996). Other plasmid manipulations were performed in *E. coli* strain DH5 α (GIBCO BRL) with standard procedures (Sambrook et al. 1989). Yeast and *E. coli* media, including the rich, glucose-containing medium (YPD), the defined media (SD with appropriate supplements), and sporulation medium were prepared by standard recipes (Sambrook et al. 1989; Rose et al. 1990). Standard procedures of yeast genetics and molecular biology were used (Guthrie and Fink 1991; Sambrook et al. 1989). Yeast transformations were performed with a modification of the Li⁺-ion method (Gietz et al. 1992). Sporulation experiments were performed essentially as described by De Virgilio et al. (1996).

DNA sequencing and sequence analyses

Sequences were obtained from double-stranded plasmid DNA, by use of the dideoxy chain termination method (Sanger et al. 1977) with Δ Tth DNA polymerase (Toyobo, Japan) and [α -³²P]dATP (Hartmann Analytic, Braunschweig, Germany). The University of Wisconsin Genetics Computer Group (GCG) programs were used to compile and analyze sequence data. Alignments were performed with the GAP, PILEUP, and PRETTY comparison programs.

Two-hybrid analysis

The interactions of Rim15p with the subunits of the Tre6P synthase complex were tested by two-hybrid analysis (Fields and Sternglanz 1994) with the LexA system described in detail elsewhere (Gyuris et al. 1993). The various full-length Tre6P synthase complex subunits were fused to the LexA DBD coding sequences in plasmid pEG202 (Zervos et al. 1993) as described by Reinders et al. (1997). Plasmid pJG4-5-*RIM15-P*, which contains a fragment of *RIM15* fused to the AD moiety of pJG4-5, was isolated in a two-hybrid screen for proteins that interact with Tps1p. Strain EGY48 (Table 1) containing the *LexAop-lacZ* reporter plasmid pSH18-34 (Gyuris et al. 1993) was co-transformed with a pEG202-derived plasmid expressing a LexA DBD fusion protein and with pJG4-5 or pJG4-5-*RIM15-P*. β -Galactosidase activities (in Miller units) were then assessed in

three independent clones of each strain grown for 16 hr at 27°C in minimal medium (containing 2% wt/vol galactose, 1% wt/vol raffinose, and 20 μ g/ml leucine).

Cloning and deletion of RIM15

The 0.9-kb *Eco*RI fragment of one *RIM15* two-hybrid library plasmid was labeled with [α -³²P]dATP (Hartmann Analytic) by use of the Prime-It II Random Primer Labeling Kit (Stratagene) and subsequently used to screen a genomic DNA library (in pSey8; kindly provided by M. Hall, Biocenter, Basel, Switzerland) as described by Sambrook et al. (1989). Three positive clones were found to contain overlapping fragments that together covered the entire *RIM15* gene. A segment of 6313 bp containing the entire *RIM15* gene including flanking sequences was sequenced on both strands (submitted as *TAK1*, EMBL database accession no. AJ001030).

The complete *RIM15* coding region was deleted by the PCR method (Wach et al. 1994) with plasmid pFA6a (*kanMX2*) as template and Taq DNA polymerase (Boehringer Mannheim). Oligonucleotides that contained 43 and 40 nucleotides immediately upstream and downstream, respectively, of the *RIM15* coding region, and 19 and 22 nucleotides upstream and downstream, respectively, of the *kanMX2* module were used to create a PCR product that contained flanking sequences of *RIM15* separated by the *kanMX2* module. This DNA was used to transform strains YEF473, PD6517, and S7-7A $\times$ S7-5A to construct AR1, NB11, and NB13, respectively (Table 1). Transformants that had *RIM15* replaced with the *kanMX2* module were identified by their geneticin-resistant growth and confirmed by PCR and/or Southern blot analysis (data not shown).

Enzyme assays and determination of metabolite levels

The activities of Tre6P synthase and neutral trehalase were measured in permeabilized cells essentially as described by De Virgilio et al. (1993, 1994). Invertase activity was determined in crude extracts according to an established protocol (Goldstein and Lampen 1975). β -Galactosidase activity was assayed using *o*-nitrophenyl- β -D-galactoside as substrate (Miller 1972). Trehalose and glycogen were measured by the procedures of De Virgilio et al. (1993) and Lillie and Pringle (1980), respectively. Protein concentrations were either measured by the Bio-Rad protein assay according to the manufacturer's instructions or by a modified Lowry assay (Peterson 1977) with BSA as standard.

Preparation and Northern analysis of RNA

Extraction of total cellular RNA was performed as described previously (Piper 1994). For Northern analysis, 10 μ g of total RNA was separated on 1.1% agarose gels containing 0.65 M formaldehyde, transferred to nitrocellulose membranes (BA 83; Schleicher and Schuell, Germany) in 20 $\times$ SSC, and hybridized with [α -³²P]dATP-labeled DNA fragments that were amplified by PCR from genomic DNA. The primers used for PCR were as follows, with the forward primers listed first and the fragment sizes generated given in parentheses: *RIM15*, 5'-CTGATTCGC-CGTCACAAGTTTGTCCACATAAGTCG-3' and 5'-CGTATTGGTAGCTGCGATAACGTCTGAAGATAATAG-3' (0.52 kb); *SSA3*, 5'-ATGTCTAGAGCAGTTGGT-3' and 5'-ATCAACCTCTTCCACTGT-3' (1.95 kb); *HSP12*, 5'-ATGTCTGACG-CAGGTAGAAAAGGATTC-3' and 5'-TTACTTCTTGGTTG-GGTCTTCTTCACC-3' (0.33 kb); *HSP26*, 5'-ATGTCATTTA-ACAGTCCATTTTGTGATTTTC-3' and 5'-TTAGTTACCCCA-CGATTCTTGAGAACAAAC-3' (0.64 kb); *UBI4*, 5'-ATGCA-GATTTTCGTCAAG-3' and 5'-GTTACCA CCCCTCAACCT-3'

(1.14 kb); *ADH2*, 5'-ATGTCTATTCCAGAACTCAAAAA-GCC-3' and 5'-TTATTTAGAAGTGTCAACAACGTATC-3' (1.05 kb); and *SSB1*, 5'-ATGGCTGAAGGTGTTTCCAAGG-TGC-3' and 5'-TTAACGAGAAGACAGGCCTTGGTGAC-3' (1.84 kb).

Plasmid constructions

For construction of the galactose-inducible *GAL1-RIM15* allele, the full-length *RIM15* coding sequence was amplified by use of the Expand Long Template PCR System (Boehringer Mannheim) and genomic DNA as template. *SalI* and *NotI* restriction sites were introduced immediately upstream of the ATG start codon and 86 bp downstream of the stop codon, respectively. This PCR product was cloned at the *SalI-NotI* site of YCpIF2 (Foreman and Davis 1994) to yield YCpIF2-*RIM15*. Plasmids YCpIF2-*GST* and YCpIF2-*GST-RIM15* were constructed by cloning of a PCR-generated *SalI-SalI* fragment, containing the 672 nucleotides downstream of and including the *GST* start codon, at the *SalI* site of YCpIF2 and YCpIF2-*RIM15*, respectively. Plasmids YCp*ADH1* and YCp*ADH1-RIM15* were constructed by replacement of the *GAL1* promoter-containing *Apal-SalI* fragments of YCpIF2 and YCpIF2-*RIM15*, respectively, with a PCR-generated *Apal-SalI* fragment containing the 854 nucleotides upstream of and including the *ADH1* start codon. Plasmids YCpIF2-*GST-RIM15*^{K823Y} and YCpIF2-*GST-RIM15*^{S709A/S1094A/S1416A/S1463A/S1661A} were constructed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with the appropriate primers that introduced the corresponding mutations and YCpIF2-*GST-RIM15* as template. All mutations introduced were confirmed by subsequent sequencing.

Protein kinase assays

GST, *GST-Rim15p*, *GST-Rim15p-K823Y*, and *GST-Rim15p-S709A/S1094A/S1416A/S1463A/S1661A* were expressed in wild-type *S. cerevisiae* cells from the *GAL1* promoter. Cells were harvested in stationary phase after growth on YP medium containing 2% galactose and 1% raffinose and disrupted by vortexing in lysis buffer [50 mM Tris-HCl at pH 7.5, 0.1 M NaCl, 1 mM EDTA, 1% NP-40, and one tablet of Complete Protease Inhibitor Cocktail (Boehringer Mannheim) per 50 ml] in the presence of acid-washed glass beads (0.4-mm diameter; Merck). The extracts were clarified three times by centrifugation at 4°C in a microfuge at 17,000 rpm, 10 min each time. The various *GST* fusions were purified from clarified cell extracts (containing ~30 mg protein per ml) after a 4-hr incubation at 4°C with glutathione-Sepharose beads (50 µl/ml; Pharmacia). Protein-bound beads were pelleted, washed four times with lysis buffer and three times with kinase buffer (50 mM Tris-HCl at pH 7.5, 20 mM MgCl₂, 1 mM DTT, 1 mM ATP, and one tablet of Complete Protease Inhibitor Cocktail per 50 ml). Kinase assays were performed at 30°C for 30 min in reaction buffer (kinase buffer containing 50 mM NaF, 10 mM Na-orthovanadate, 15 mM p-NO₂-phenylphosphate, 50 mM β-glycerophosphate, 5 mM Na-pyrophosphate, 10 µCi [γ-³²P]ATP, and, where indicated, 250 µg/ml α-casein). Reactions were stopped by adding SDS-gel loading buffer and boiling for 5 min and were then subjected to SDS-PAGE. Gels were dried and exposed to X-ray film or to a PhosphorImager. Quantitation of ³²P was performed with the Phosphor Analyst software (Bio-Rad).

To assay *in vitro* autophosphorylation and phosphorylation of Rim15p by bovine cAPK, wild-type or mutant *GST-Rim15p* fusion protein was purified as described above. Equal amounts of protein-bound beads (10 µl) were resuspended in reaction buffer and incubated for 30 min at 30°C in the presence or

absence of 1 unit of A kinase catalytic subunit (Sigma) and/or A kinase Inhibitor (Sigma). Reactions were stopped and analyzed as described above. When the effects of cAPK-dependent phosphorylation on Rim15p activity were monitored, reactions were incubated in [γ-³²P]ATP-free reaction buffer in the presence or absence of 1 unit A kinase catalytic subunit, subsequently terminated by three washes with the same buffer (without A kinase catalytic subunit), and then the mixtures were incubated with α-casein and [γ-³²P]ATP as described above.

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