Für Claudio –
Merci für alles!
Anke

TREHALOSE SYNTHESIS AND NUTRIENT SIGNALING IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumine
cAMP	adenosine 3'-5'-cyclic monophosphate
cAPK	cAMP-dependent protein kinase
cDNA	complementary desoxyribonucleic acid
DEPC	diethylpyrocarbonate
DMF	dimethylformamide
DNA	desoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(2-aminoethyl) tetraacetic acid
EMBL	European Molecular Biology Laboratory
Fru6P	fructose-6-phosphate
GAP	GTPase-activating protein
GEF	GTP-exchange factor
Glu6P	glucose-6-phosphate
GST	glutathione S-transferase
GTP	guanidine 5'-triphosphate
HPLC	high performance liquid chromatography
HSE	heat-shock element
HSF	heat-shock factor
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilo bases (*i.e.* 1'000 nucleotides)
MBP	maltose binding protein
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium salt
OD	optical density
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
STRE	stress-response element

TBS	Tris-buffered saline
Tre6P	trehalose-6-phosphate
Tricine	N-tris(hydroxymethyl)-methylglycine
Tris	tris(hydroxymethyl)-aminomethane
UDP	uridine 5'-diphosphate
X-phosphate	5-bromo-4-chloro-3-indolyl phosphate toluidinium salt

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SUMMARY

The non-reducing disaccharide trehalose is widespread in nature and in many organisms its synthesis has been found to be induced in response to a small set of specific environmental conditions. In yeast trehalose is accumulated during nutrient starvation, desiccation, and after exposure to a mild heat shock. Therefore, trehalose has been suggested to act as a stress protectant. Synthesis of trehalose in the yeast *Saccharomyces cerevisiae* is a two-step process that is catalyzed by the trehalose-6-phosphate (Tre6P) synthase/phosphatase complex. This complex is composed of at least three subunits, encoded by the genes *TPS1*, *TPS2*, and *TSL1*. The *TPS3* gene, a homolog of *TSL1*, was identified through systematic sequencing but at the present time, has not been shown to constitute a fourth subunit of the Tre6P synthase/phosphatase complex. Besides its role in trehalose synthesis, Tps1 was also found to be important for the regulation of the glycolytic pathway. The mechanisms by which Tre6P synthase activity is regulated are unclear. Interestingly however, it has been suggested that Tre6P synthase may be postranslationally regulated by cAMP-dependent phosphorylation, as has been previously shown for the trehalose-degrading neutral trehalase. The cAMP-dependent protein kinase (cAPK) is the central enzyme of the Ras/cAMP pathway in *S. cerevisiae*, which is required for proper regulation of growth, cell cycle progression and development in response to nutritional conditions. By identifying regulators of Tps1 it may therefore be possible to establish a link between trehalose synthesis, nutrient signaling, and regulation of glycolysis. The central focus of the present PhD thesis has therefore been the detailed characterization of the Tre6P synthase/phosphatase complex as well as the identification of proteins with a regulatory role in trehalose synthesis.

The results of the experimental work are presented in three chapters:

CHAPTER I describes how the two-hybrid system was utilized to directly assay the physical interactions of Tps1, Tps2, Tps3, and Tsl1 *in vivo*. Additionally, physiological experiments were performed with a set of isogenic yeast mutants deleted for the potential subunits in all possible combinations. With these two approaches it was possible to show (i) that Tps1, Tps2, Tps3, and Tsl1 interact *in vivo*, demonstrating for the first time that Tps3 is part of the complex; (ii) that Tps1, Tps2, and Tps3 are able to form homodimers; (iii) that Tps1 and Tps2 carry the catalytic activities of Tre6P synthase and Tre6P phosphatase, respectively; and (iv) that Tsl1 and Tps3 might share a common function with respect to regulation and/or stabilization of the Tre6P synthase/phosphatase complex. This chapter has been published in *Molecular Microbiology* **24**: 687-695 (Reinders *et al.*, 1997). This project was a collaboration with the group of Johan

Thevelein (Katholieke Universiteit Leuven, Belgium) and a second publication resulting from this collaboration was recently submitted to *The Journal of Biological Chemistry* (Bell *et al.*, 1998).

Based on the knowledge gained in CHAPTER I, CHAPTER II describes how a two-hybrid screen for regulatory proteins of the Tre6P synthase led to the identification of a new protein kinase gene, *RIM15*. Interestingly, one of the phenotypes caused by loss of *RIM15* was the deficiency of trehalose accumulation in stationary phase. In addition, stationary *rim15Δ* cells were also defective for the induction of a number of adaptations usually induced in response to nutrient limitation. These include the accumulation of glycogen, proper G_1 arrest, acquisition of stress resistance, and transcriptional derepression of *SSA3*, *HSP12*, and *HSP26*. An interesting additional phenotype of diploid *rim15Δ* cells was their sporulation defect. Together, these phenotypic traits of *rim15Δ* cells resembled those caused by mutations which result in hyperactive cAPK (*e.g. bcy1, RAS2*^{val19}). In contrast to these mutants, however, deletion of *RIM15* had no measurable defect in glucose repression/derepression. Taken together the detailed physiological investigations of a *rim15Δ* mutant presented in this chapter, indicate that Rim15 may be an element of the Ras/cAMP or a related nutrient signaling pathway.

The hypothesis that Rim15 may be an element of the Ras/cAMP pathway was further examined in CHAPTER III. Genetic analyses demonstrated that *rim15Δ* is epistatic to *cdc35-10* and complete loss of cAPK activity. In support of this notion, overexpression of *RIM15* suppressed several phenotypes of *bcy1-1*, exacerbated the growth defect of a temperature-sensitive *cdc35-10* strain, and partially induced a starvation response in exponentially growing wild-type cells. Intriguingly, kinase assays with epitope-tagged Rim15 provided evidence that Rim15 has protein kinase activity that can be inhibited by prior *in vitro* phosphorylation of Rim15 by cAPK. This result marks Rim15 as the first protein kinase identified that may be a downstream target of cAPK. Finally, the possible role of Rim15 as a potential regulator of Tre6P synthase activity was investigated by kinase assays and co-precipitation studies. Surprisingly, no evidence for a phosphorylation of Tps1 by Rim15 could be obtained so far and the regulatory role of Rim15 with respect to trehalose synthesis remains to be elucidated as will be discussed in more detail in this chapter. In sum, the results obtained are consistent with a model in which Rim15 constitutes an element of the Ras/cAMP pathway, acting downstream and under negative control of cAPK to control entry into stationary phase, including the accumulation of trehalose. Some of the results presented in CHAPTER II and III are part of a manuscript (Reinders *et al.*, 1998) that has been submitted to *Genes & Development* (see first page of these chapters for details).

GENERAL INTRODUCTION

Following the Ph.D. projects of Thomas Hottiger (1988), Walter Bell (1992), and Claudio De Virgilio (1993), this has been the fourth Ph.D. thesis at the Botanical Institute dealing with aspects of trehalose synthesis in yeast. With a plausible concept for the role of trehalose as a stress protectant worked out previously (T. Hottiger and C. De Virgilio), and the structural genes coding for the enzymes responsible for trehalose synthesis in *Saccharomyces cerevisiae* identified (W. Bell and C. De Virgilio), I set out in 1994 to further investigate the regulation of trehalose synthesis. In the course of my thesis work the scope of the project broadened and it now also includes aspects of nutrient signaling in *S. cerevisiae*.

This General Introduction explains occurrence, synthesis, and catabolism of trehalose, and its role in stress protection. It also describes the unexpected finding of an additional role of Tps1, the Tre6P synthase, in the regulation of glycolysis. As will be explained further, one of the conditions under which trehalose synthesis is induced in *S. cerevisiae* is under conditions of nutrient limitation, that is, upon entry into stationary phase. It can be assumed that these two events, namely trehalose accumulation and the induction of a quiescent state, must be regulated by nutrient signaling pathways. Consequently, this General Introduction also describes stationary phase as a distinct state apart from the cell cycle, the Ras/cAMP pathway, as an important nutrient signaling pathway involved in the cells' decision to enter stationary phase, and the possible role of this pathway in regulating trehalose metabolism.

Occurrence of trehalose

The common name trehalose for α-D-glucopyranosyl α-D-glucopyranoside is derived from trehala manna, the cocoons of *Larinus nidificans,* a desert weevil from Asia minor (Berthelot, 1858, cited in Becker *et al.*, 1996). For the first time trehalose has probably been isolated from the ergot of rye, therefore it was also named mycose (Wigger, 1832, cited in Elbein, 1974). Since then this disaccharide has been found in a wide range of different organisms ranging from bacteria to fungi, plants, and lower animals (Elbein, 1974). Interestingly, many of these organisms are anhydrobiotic, which means that they are capable of withstanding extreme desiccation during drought and to recover completely upon rehydration. In the dry state these organisms contain high amounts of trehalose and they can remain metabolically inert for years. Resumption of metabolic activity is usually accompanied by degradation of trehalose. Known examples of anhydrobiotic organisms are baker's yeast (*S. cerevisiae*), but also so-called resurrection plants like the 'Rose of

Jericho' (*Selaginella lepidophylla*), a pteridophyte growing in Mexico, the brine shrimp (*Artemisia salina*), and certain nematodes (for a review on anhydrobiosis see Crowe *et al.*, 1992). Trehalose is also the main haemolymph sugar in insects, providing energy for the flight muscle (Becker *et al.*, 1996). In higher plants, the occurrence of trehalose was only reported from a single species, *Myrothamnus flabellifolia*, a plant found in South-Western Africa (Bianchi *et al.*, 1993; Drennan *et al.*, 1993; Müller *et al.*, 1995). Recent publications reporting the occurrence of trehalose synthesis genes and even low amounts of trehalose in angiosperms indicates that trehalose may be more widely spread also in higher plants (Goddijn *et al.*, 1997; Vogel *et al.*, 1998; Blázquez *et al.*, 1998).

Synthesis of trehalose

In yeast trehalose is synthesized by a two-step process in the cytosol, the location of trehalose storage (Keller *et al.*, 1982). In a first step, trehalose-6-phosphate (Tre6P) is formed from glucose-6-phosphate (Glu6P) and UDP-glucose (UDPG), catalyzed by the enzyme Tre6P synthase (EC 2.4.1.15). In a subsequent reaction, the phosphate is cleaved off by a highly specific phosphatase, the Tre6P phosphatase (EC 3.1.3.12). The process was first described by Cabib and Leloir (1958). This type of trehalose synthesis has also been found in many other trehalose accumulating organisms, for instance other yeasts (Vicente-Soler *et al.*, 1989; Luyten *et al.*, 1993; Blázquez *et al.*, 1994), filamentous fungi (Borgia *et al.*, 1996; Wolschek and Kubicek, 1997), slime molds (Roth and Sussman, 1966; Roth and Sussman, 1968), bacteria (Giæver *et al.*, 1988; Kaasen *et al.*, 1992), and insects (Becker *et al.*, 1996, and references cited herein). No trehalose synthesizing enzymes have been studied in detail or even purified from plants. Since it was reported that genes from *Arabidopsis thaliana* with homology to *S. cerevisiae* Tre6P synthase and Tre6P phosphatase genes were able to complement yeast mutants defective for Tre6P synthase or Tre6P phosphatase, respectively, it seems possible that the same mode of trehalose synthesis is conserved between plants and *S. cerevisiae* (Blázquez *et al.*, 1998; Vogel *et al.*, 1998).

In *S. cerevisiae*, several studies have dealt with the purification and kinetic properties of trehalose synthesizing enzymes (Cabib and Leloir, 1958; Elander, 1968; Vandercammen *et al.*, 1989; Bell *et al.*, 1992; Londesborough and Vuorio, 1991; Londesborough and Vuorio, 1993; De Virgilio *et al.*, 1993). Early on it had been found that the two enzymatic activities, Tre6P synthase and Tre6P phosphatase, copurified. Vandercammen *et al.* (1989) had therefore suggested that trehalose synthesis might be catalyzed by a bifunctional enzyme. However, the general picture emerging from the purification studies is that Tre6P synthase and Tre6P phosphatase are two subunits of a large multimeric protein complex. This protein complex consists of at least three different subunits. It has an esti-

mated size of 630-800 kDa. During purification it was found to be rather susceptible to proteolysis. Some variance existed therefore between the size determinations of the single subunits made by different groups. Isolation and subsequent sequencing of the genes coding for these subunits has eventually resolved this particular matter of debate (see below).

In the initial investigation of the biochemical properties of the partially purified Tre6P synthase/phosphatase, Cabib and Leloir (1958) had determined a pH optimum of 6.6 in the presence of 25 mM Mg^{2+}. Kinetic studies of different groups working with Tre6P synthase/phosphatase complexes purified to various degrees revealed approximate K_m values for the Tre6P synthase of 3.5 mM for Gluc6P and 0.5 mM for UDPG, strong inhibition by phosphate and stimulation by fructose-6-phosphate (Fru6P; Elander, 1968; Vandercammen *et al.*, 1989; Londesborough and Vuorio, 1991; Bell *et al.*, 1992; Londesborough and Vuorio, 1993). The K_m value determined for the Tre6P phosphatase was 0.2 mM Tre6P at pH 6.0, the optimal pH for activity. In addition, Tre6P phosphatase was also found to be completely Mg^{2+} dependent and activated by phosphate (Vandercammen *et al.* 1989; Londesborough and Vuorio, 1991; Londesborough and Vuorio, 1993).

It is known that trehalose levels vary considerably during the life cycle of *S. cerevisiae* (see also below). While cells growing rapidly on fermentable carbon sources (*e.g.* glucose) usually do not accumulate trehalose, they contain high levels of it in stationary phase (Lillie and Pringle, 1980). Yeast spores are also known to hold large amounts of trehalose (Roth, 1970). It is therefore reasonable to assume that trehalose synthesis and the enzymes responsible for this process should be tightly regulated. Several different models for the regulation of trehalose synthesis have been discussed in the literature. These include both transcriptional and posttranslational (*i.e.* regulation by chemical or physical effectors, by availability of substrates, by limited proteolysis, and by phosphorylation/dephosphorylation) control mechanisms. These particular regulatory aspects of trehalose synthesis will be dealt with in more detail in the Introduction of Chapter II.

The genes coding for the known subunits of the Tre6P synthase/phosphatase complex in *S. cerevisiae* have been identified. *TPS1* (also called *TSS1* by Vuorio *et al.*, 1993) was cloned with the help of antibodies raised against the purified protein and codes for the small subunit. The ORF is 1485 base pairs long, corresponding to 495 amino acids, and predicted to encode a 56 kDa protein, which is in good agreement with size estimations from purification studies (Bell *et al.*, 1992; Vuorio *et al.*, 1993). Surprisingly, the sequence of *TPS1* was found to be almost identical to the previously sequenced *CIF1*, a gene essential for growth on glucose (Gonzáles *et al.*, 1992). Subsequently, it has been shown that a number of mutations described earlier as *byp1* (Breitenbach-Schmitt *et al.*, 1984), *cif1* (Navon *et al.*, 1979), and *fdp1* (van de Poll *et al.*, 1974), and more recently *ggs1* (Van Aelst *et al.*, 1993), and *glc6* (Cannon *et al.*, 1994) are all alleles of the same

gene. Accordingly, the corresponding DNA sequences, *CIF1* (Gonzáles *et al.*, 1992), *GGS1* (Van Aelst *et al.*, 1993), and *GLC6* (Cannon *et al.*, 1994) are virtually identical to *TPS1*. Deletion of *TPS1* causes a highly pleiotropic phenotype, including the inability to grow on rapidly fermentable carbon sources and the lack of trehalose synthesis. These discoveries had motivated Bell *et al.* (1992) to suggest that Tps1 might not be the Tre6P synthase itself but rather a regulatory protein. In the meantime, however, several lines of evidence, in addition to the fact that *tps1Δ* strains display no Tre6P synthase activity (Bell *et al.*, 1992), have been presented that make it very likely that Tps1 is indeed the Tre6P synthase. Accordingly, expression of *TPS1* in *E. coli* complemented the Tre6P synthase deficient *otsA* mutant (McDougall *et al.*, 1993), overexpression of *TPS1* in *E. coli* led to increased Tre6P synthase activity (Vuorio *et al.*, 1993), and transgenic tobacco plants expressing *TPS1* were able to synthesize trehalose (Holmström *et al.*, 1996). The potential role of Tps1 in the regulation of glycolysis will be discussed in more detail below. The 100 kDa-subunit, Tps2, is encoded by the *TPS2* gene. The ORF is 2691 base pairs long, corresponding to 897 amino acids, with a predicted molecular weight of 102.8 kDa (De Virgilio *et al.*, 1993). *TPS2* codes for the Tre6P phosphatase, deletion of the gene results in accumulation of Tre6P under conditions where the wild-type strain accumulates trehalose and leads to temperature sensitivity (De Virgilio *et al.*, 1993). *TPS2* is also allelic to *HOG2*, a gene whose mutation confers an osmosensitive phenotype and to *PFK3,* a gene identified in a screen for mutations that prevent growth on glucose in a *pfk1* (coding for a subunit of phosphofructokinase) background (Brewster *et al.*, 1993; Sur *et al.*, 1994). Diploid *tps2* cells are unable to sporulate (Sur *et al.*, 1993). The third known subunit, Tsl1, is encoded by the *TSL1* gene (Vuorio *et al.*, 1993). The ORF was found to be 3294 base pairs long, corresponding to 1098 amino acids, and encoding a protein with a predicted molecular weight of 123 kDa. No enzymatic function could be assigned to this protein but the fact that limited proteolytic degradation of Tsl1 increased the activity of the Tre6P synthase/phosphatase complex and caused insensitivity to the allosteric regulators Fru6P and phosphate (see above) makes it likely that Tsl1 has regulatory functions (Londesborough and Vuorio, 1991; Vuorio *et al.*, 1993).

Remarkably, *TPS1* shows striking homology to both *TPS2* and *TSL1* over its entire sequence (30% identity over the entire amino acid sequence of *TPS1* upon optimal alignment). The significance of this finding is unclear. A fourth gene, *TPS3*, with significant homology to *TSL1* (55% identity upon optimal alignment of the two genes over their entire amino acid sequences) has been identified by systematic sequencing (Manning *et al.*, 1992), but has not been shown to be part of the Tre6P synthase/phosphatase complex (see also Chapter I). This gene predicts a protein of 1055 amino acids with a deduced molecular weight of 115 kDa.

Catabolism of trehalose

In yeast and other fungi trehalose is degraded by the enzyme trehalase (EC 3.2.1.28) that cleaves trehalose into two glucose molecules. Trehalase was first described by Bourquelot (1832, cited in Elbein, 1974). Since that time trehalase activity has been detected in a wide range of organisms, including some which neither synthesize nor store trehalose. Examples of such sources are the small intestine and the renal brush-border of mammals (Ishihara *et al.*, 1997) and many higher plants (reviewed in Müller *et al.*, 1995; see also above). In mammals trehalase activity seems to be important for the hydrolysis of ingested trehalose, but the role of trehalase in plant tissues is unclear. Trehalase from all sources is effectively inhibited by validamycin, a structural analog of trehalose (Asano *et al.*, 1990).

Because it was observed that yeast cells contained high amounts of trehalose in stationary phase and simultaneously displayed high trehalase activity, it was suspected that trehalose and trehalase might be in different compartments. After careful preparation of vacuoles, Keller *et al.* (1982) were indeed able to demonstrate that an acid trehalase with a pH optimum of 5.5 was confined to the vacuoles while trehalose was located in the cytoplasm. However, the same group also showed the occurrence of 'trehalase zymogen' in the cytoplasm, measurable after activation with cAMP and ATP *in vitro* (Wiemken and Schellenberg, 1982). They initially concluded that the 'trehalase zymogen' may be a precursor of the vacuolar trehalase that is transported to the vacuole after phosphorylation. In 1984, Londesborough and Varimo (1984) then purified two separate trehalases with distinctly different properties from *S. cerevisiae*. They called them V-trehalase and C-trehalase, according to their location in the vacuole and in the cytoplasm. Because of their different pH optima these trehalases are now usually referred to as acid and neutral trehalase.

Acid trehalase

Acid trehalase has been detected in many fungi (reviewed in Thevelein, 1984b). As described above, in *S. cerevisiae* it is located in the vacuole. In accordance with this, Londesborough and Varimo (1984) had found that this trehalase binds strongly to Concanavalin A, suggesting that it is a glycosylated protein. This has later been confirmed by Mittenbühler and Holzer (1988) who determined a carbohydrate content of 86% for acid trehalase. In accordance with the vacuolar localization it was also found that defects in the secretory pathway lead to a decrease of vacuolar trehalase activity while the cytosolic activity of neutral trehalase was not affected (Harris and Cotter, 1987). The same authors could also demonstrate that maturation of a precursor in the vacuole depends upon proteinase A (Harris and Cotter, 1988). The size of glycosylated acid trehalase was estimated to be 210-215 kDa (Londesborough and Varimo, 1984; Mittenbühler and Holzer, 1988)

by gel filtration. The partially purified enzyme had a pH optimum of 4.0 - 5.0 and a K_m of 1.4 mM for trehalose, it was activated about 30% in the presence of 100 mM KCl and inhibited by acetic acid with a $K_i \approx 15$ mM (Londesborough and Varimo, 1984). The gene encoding acid trehalase, *ATH1*, has been cloned and it predicts a protein of 117 kDa (Destruelle *et al.*, 1995). Because some doubt remained as to whether the identified gene was indeed the structural gene of acid trehalase, Alizadeh and Klionsky (1996) purified acid trehalase from a strain overexpressing *ATH1* and subjected it to peptide analysis. The peptide sequences were found to match the deduced amino acid sequence of *ATH1*. Finally, it could be shown that deglycosylated Ath1 has a size of 85 kDa, which suggests that a 30 kDa precursor sequence was removed during processing of acid trehalase in the vacuole (Alizadeh and Klionsky, 1996).

The role of acid trehalase is not clearly understood. The enzyme activity can only be detected in glucose-grown resting cells or in cells growing on non-fermentable carbon sources like ethanol or glycerol (San Miguel and Argüelles, 1994). On glucose, acid trehalase activity is repressed. This pattern makes it unlikely that acid trehalase is involved in the degradation of stored trehalose. Nevertheless, loss of *ATH1* led to higher accumulation of trehalose and rendered cells more resistant to dehydration, freezing, and ethanol stress (Kim *et al.*, 1996). Moreover, it has been suggested that acid trehalase may be important for the growth of *S. cerevisiae* on trehalose as carbon source. The enzymatic activity was shown to be induced during growth on trehalose and cells lacking *ATH1* were unable to grow on trehalose as the sole carbon source (Nwaka *et al.*, 1996). It remains unclear how in this case the enzyme would gain access to its substrate. The authors propose that trehalose could be taken up by the trehalose transporter described by the Panek group (Eleutherio *et al.*, 1993) but no explanation has been given as to how the sugar would reach the vacuole. More work needs to be done in order to reach a better understanding of the role of acid trehalase in *S. cerevisiae*.

Neutral trehalase

Neutral trehalase has also been detected in several fungal species, but it seems to be less common than acid trehalase (reviewed in Thevelein, 1984b). As mentioned above, the enzyme is located in the cytoplasm. Londesborough and Varimo (1984) partially purified a 170 kDa protein with a pH optimum of 6.7 and a K_m of 5.7 mM for trehalose. The enzyme was dependent on the presence of Ca^{2+} or Mn^{2+}, inhibited about 65% by 100 mM KCl and strongly inhibited by 0.1 mM $ZnCl_2$. An interesting feature of neutral trehalase is that it is activated by phosphorylation. Early reports had already provided evidence for an involvement of cAMP in the regulation of neutral trehalase (van der Plaat, 1974; van Solingen and van der Plaat, 1975). Since then several authors have reported that the enzyme can be phosphorylation-activated *in vitro* by addition of the necessary

agents (Wiemken and Schellenberg, 1982; Ortiz *et al.*, 1983; Thevelein, 1984a). Uno *et al.* (1983) convincingly demonstrated that the conversion of neutral trehalase from the inactive to the active form is achieved through phosphorylation by cAPK. Increased activation of purified trehalase *in vitro* resulted in the phosphorylation of a 80 kDa protein. Indeed, App and Holzer (1989) found the purified active neutral trehalase to be a dimer, consisting of two identical subunits of 80 kDa. Their analysis of the biochemical properties of purified neutral trehalase were in good agreement with those determined by Londesborough and Varimo (1984) with the partially purified enzyme. However, the K_m of the purified enzyme was found to be 34.5 mM trehalose (App and Holzer, 1989). Accordingly, neutral trehalase displays a much lower affinity to trehalose than acid trehalase. While activation was observed following phosphorylation, it was also shown that treatment with alkaline phosphatase resulted in inactivation of neutral trehalase (Ortiz *et al.*, 1983; Uno *et al.*, 1983; App and Holzer, 1989). It was suggested that the neutral trehalase activity is also controlled by phosphorylation/dephosphorylation *in vivo* since the strong decrease of trehalase activity upon the diauxic shift correlated with a decrease in the phosphorylated form of neutral trehalase (Coutinho *et al.*, 1992).

The gene coding for neutral trehalase, *NTH1*, has been cloned. It was found to have homology to the periplasmatic trehalase from *E. coli* and to the trehalase gene of rabbit small intestine. The gene also contains consensus sites for cAMP-dependent phosphorylation (Kopp *et al.*, 1993; Kopp *et al.*, 1994). Upon heat shock, yeast cells in log-phase are known to accumulate trehalose (Hottiger *et al.*, 1989; see also below). This trehalose is mobilized rapidly after downshift of the cultures to lower temperatures, due to the activation of neutral trehalase (De Virgilio *et al.*, 1991b). Deletion of *NTH1* led to slightly higher amounts of trehalose in log-phase cells subjected to a heat shock at 40°C. However, upon shift to 27°C the *nth1Δ* mutant was severely delayed in trehalose mobilization and concomitantly retained higher thermotolerance (De Virgilio *et al.*, 1994). During the growth cycle the pattern of activity of neutral trehalase was found to be opposite to the pattern observed for acid trehalase. Neutral trehalase was active during exponential growth on fermentable carbon sources but its activity sharply declined during the diauxic shift in parallel with the onset of trehalose synthesis. The enzyme was reactivated by addition of fresh growth medium to stationary phase cells (San Miguel and Argüelles, 1994). This is in agreement with neutral trehalase being the main activity responsible for trehalose mobilization in yeast. Neutral trehalase has also been implicated in being important for the recovery of yeast cells after heat shock (Nwaka *et al.*, 1995a).

Recently a gene with high homology to *NTH1* (77% identity at the amino acid level) has been identified. It was named *NTH2*, but so far it has not been shown that the corresponding protein is indeed another trehalase and deletion of *NTH2* did not lead to a loss of trehalase activity in the cells (Nwaka *et al.*, 1995a; Nwaka *et al.*, 1995b).

Physical properties of trehalose and its role in cell protection

At the beginning of this chapter it has been described that trehalose is found in a large number of drought-resistant organisms. Moreover, it could be shown that the accumulation of trehalose was directly correlated with the ability of these organisms to survive complete dehydration (Crowe *et al.*, 1984; for reviews see Wiemken, 1990; Crowe *et al.*, 1992). The biophysical basis for this astonishing property of trehalose to protect organisms from the adverse effects of water loss, has been studied in much detail. As a result, two functions of trehalose have been proposed, namely the stabilization of biological membranes and the protection of proteins.

Stabilization of membranes

Drying of biological membranes leads to destructive events including fusion and liquid crystalline to gel phase transitions. As one consequence of these events, membranes become leaky, resulting in uncontrolled efflux and influx of substances. A second consequence is the irreversible aggregation of membrane components (Crowe *et al.*, 1992).

The ability of trehalose to stabilize biological membranes has been investigated using liposomes as a model system. Indeed, during freeze-drying, trehalose protected the integrity of the liposomes, as seen by the absence of leakage or fusion (Crowe *et al.* 1985, for a review see Crowe *et al.*, 1992). Moreover, it has been demonstrated that trehalose is superior in this respect to other sugars like sucrose because it was far more effective at lower concentrations (Crowe *et al.*, 1987). As a molecular basis for the observed protective effect, the water-replacement hypothesis has been developed. This hypothesis proposes that trehalose is able to directly interact by hydrogen-bonding with the polar head-groups of membrane phospholipids, thereby effectively replacing the water around these charged groups and preventing the damaging phase transitions both during drying and rehydration (Crowe *et al.*, 1984; Crowe *et al.*, 1988).

Protection of proteins

Many forms of physiological stress directly affect cellular proteins. For example, heat stress leads to conformational changes of proteins that are often irreversible. Trehalose has been shown to be able to protect the native conformation of proteins *in vitro* during conditions of heat, freezing, and desiccation. Addition of trehalose to enzymes like Glu6P dehydrogenase, pyrophosphatase, or to the restriction enzyme *Eco*RI prior to incubation at high temperatures preserved their *in vitro* activities (Hottiger *et al.*, 1994;

Sola-Penna and Meyer-Fernandez, 1994). Trehalose also prevented the irreversible dissociation of phosphofructokinase into inactive dimers during drying or freezing (Carpenter *et al.*, 1987; Crowe *et al.*, 1992). In order to explain the preserving effect of trehalose on proteins it has been suggested that the property of trehalose to undergo glass rather than crystal formation upon desiccation would result in the establishment of a physical state that is particularly protective for macromolecules. However, that would not explain the protection of proteins during heat treatment (reviewed in Thevelein, 1996). A second hypothesis by Timasheff (1993) explains that the addition of a sugar to a protein generally creates a thermodynamically unfavorable situation, since it increases the chemical potentials of both agents. Denaturation would exacerbate this unfavorable situation because it would increase the contact surface between protein and sugar, therefore the native state of the protein would be the preferred state. This explanation, however, would not explain the superiority of trehalose over other sugars.

The reasons for the efficiency of trehalose in protecting cellular structures and functions may lie in the special physical and chemical properties of this sugar. These include very high hydrophilicity, nonhygroscopic glass formation, very high chemical stability, nonreducing character, and an unusual flexibility of the disaccharide bond. But even though the protective effect of trehalose is not completely understood at the molecular level, it has been employed quite effectively even in industrial processes apart from the yeast industry (see also below). Trehalose is added to preparations in order to preserve or to extent the shelf life of pharmaceuticals, enzymes and even living microorganisms, *e.g. E. coli* (Blakeley *et al.*, 1990; Louis *et al.*, 1994; Panek, 1995; Thevelein, 1996 and references therein; Carninci *et al.*, 1998). Most interestingly, trehalose is now also used in the food industry because of its astonishing capabilities to preserve the structure and taste of dried foodstuffs (Portman and Birch, 1995; Panek, 1995 and references cited in these publications).

The role of trehalose in protection of S. cerevisiae *cells*

For baker's yeast cells it has been known for a long time that they accumulate trehalose (Koch and Koch, 1925, cited in De Virgilio, 1993), but originally it had been regarded as a storage compound like glycogen. The suggestion that trehalose may also fulfill an additional role as a stress protectant in this organism has been a rather recent one (Van Laere, 1989; Wiemken, 1990).

On the one hand this suggestion stems from the observation that trehalose, not only in yeast but also in other fungi, is mainly found during developmental stages and growth phases that are desiccation and heat tolerant, like spores, conidia, and sclerotia or stationary phase cells (Küenzi and Fiechter, 1972; Grba *et al.*, 1975; Emyanitoff and Wright,

1979; Thevelein, 1984a; Martín *et al.*, 1986). On the other hand, a closer examination of the pattern of trehalose accumulation in *S. cerevisiae* revealed that it did not show the usual pattern of accumulation expected from a mere storage carbohydrate. Such a reserve compound would be expected to become accumulated in times of plenty and to be consumed in times of need. Glycogen, as the other abundant carbohydrate present in yeast cells, fulfills these criteria. When both glycogen and trehalose amounts were measured under different nutrient limiting conditions, it was noted that their patterns of accumulation and utilization were not identical (Lillie and Pringle, 1980). On rich glucose-based medium glycogen accumulation was found to begin during exponential growth at a time when about half of the glucose had been consumed. It was then partially mobilized during the diauxic shift, probably to provide energy during respiratory adaptation but possibly also to synthesize trehalose. During prolonged starvation in stationary phase, glycogen was degraded (Lillie and Pringle, 1980). Trehalose, on the other hand, has been observed to accumulate only when glucose has been exhausted from the growth medium, which also coincided with a shift in glycogen metabolism from net synthesis to net degradation, so that no net increase in storage carbohydrate content was achieved (Lillie and Pringle, 1980). The same mechanism also seems to be in effect during sporulation (Kane and Roth, 1974). In contrast to the breakdown of glycogen, appreciable trehalose breakdown was observed to occur only very late in stationary phase (>130 d), and even then only very slowly. Coinciding with trehalose mobilization was a rapid loss of viability of the cells (Lillie and Pringle, 1980). In contrast, starved cells or yeast spores that were incubated in the presence of a fermentable carbon source, rapidly mobilized their accumulated trehalose. At least in the case of germinating spores, this mobilized trehalose is then apparently not used as an energy source. Instead, it has been observed that germinating spores secreted glucose into the medium (Thevelein, 1984b; Van Laere, 1989; Wiemken, 1990). Apart from these findings, it has also been pointed out that from an energetic viewpoint trehalose accumulation is less advantageous than the accumulation of glycogen, since it not only costs more energy to synthesize but even yields less energy upon mobilization (Wiemken, 1990).

Taken together, these observations, namely (i) that trehalose occurs in a number of anhydrobiotic organisms, (ii) that trehalose can protect biological membranes and proteins *in vitro*, and (iii) that the pattern of accumulation of trehalose in *S. cerevisiae* is different from the pattern of accumulation of the storage carbohydrate glycogen, strongly suggested that trehalose has an additional role in protection of *S. cerevisiae*. Further support for this suggestion was provided by the finding that yeast cells also accumulated trehalose in response to elevated temperatures (Grba *et al.*, 1975). Since then, intensive efforts by many labs around the world, studying a number of different microorganisms, have provided us with solid evidence that trehalose is indeed a stress protectant *in vivo*. The following paragraphs will summarize some of the results corroborating this special role of

trehalose under conditions of heat, desiccation, freezing, and osmotic stress, mainly focusing on *S. cerevisiae*, the organism under study in the present Ph.D. thesis.

The above mentioned observation that yeast cells accumulate trehalose when incubated at higher temperatures has been reported in a number of studies. Hottiger *et al.* (1987) could show that the amount of trehalose increased rapidly when cells were shifted from 27°C to 40°C. Moreover, this increase in trehalose content was correlated with an increase in thermotolerance. While control cells at 27°C did not survive a challenging heat shock of 8 min at 50.4°C, about 50% of the cells incubated at 40°C for 1 h (during which the cells accumulated high amounts of trehalose) prior to the challenging heat shock survived the subsequent challenging heat shock. The same authors also observed a strong correlation between trehalose content and thermotolerance in mutants in the Ras/cAMP pathway (see also below) that contained different amounts of trehalose (Hottiger *et al.*, 1989). A study by Attfield *et al.* (1992), using a set of related, unmutated yeast strains that accumulated varying amounts of trehalose, confirmed the correlation. However, all of these studies were hampered by the fact that under the conditions used, not only trehalose synthesis was induced but also the synthesis of heat-shock proteins (hsps). The induction of synthesis of this special set of proteins is a hallmark of the heat-shock response, a molecular reaction to stressful but sublethal temperature, conserved among all organisms. Hsps have also been implicated in protecting the cells from the damaging effects of elevated temperatures, for instance by acting as chaperones, preventing heat-induced misfolding of cellular proteins (Plesofsky-Vig, 1996). So far it has only been demonstrated for a limited number of hsps, for instance Hsp104, Hsp70, Ctt1, and Ubi4 that they have an appreciable effect on the induction of thermotolerance (Finley *et al.*, 1987; Wieser *et al.*, 1991; for reviews see Piper, 1993; Mager and Ferreira, 1993; Parsell *et al.*, 1994). In order to be able to distinguish between the possible roles of trehalose and hsps, some researchers chose experimental conditions which allowed trehalose but restricted protein synthesis. When the translation inhibitor cycloheximide was added to *S. cerevisiae* cultures prior to a heat shock or cells of a temperature-sensitive yeast strain were incubated at the nonpermissive temperature for protein synthesis (40°C, as determined by incorporation of [^{3}H] isoleucine into TCA-precipitable material), cells were still able to accumulate trehalose and to acquire thermotolerance, albeit to a lesser extent (Hottiger, 1988; De Virgilio *et al.*, 1991b; Neves and François, 1992). The identification of the structural genes of the trehalose synthesizing enzymes in baker's yeast as well as the genes of hsps made it possible to genetically assess the contribution of trehalose *versus* hsps for the acquisition of thermotolerance. Convincing genetic evidence that heat-induced trehalose synthesis is an important factor for thermotolerance induction, came from a study using strains deleted for *TPS1* (encoding the Tre6P synthase), *TPS2* (encoding the Tre6P phosphatase) and *NTH1* (encoding the neutral trehalase). Both strains unable to accumulate trehalose, *tps1Δ* and *tps2Δ*, were less thermotolerant after a preconditioning

heat shock, when compared with the wild-type strain. In contrast, the *nth1Δ* mutant, defective for mobilization of trehalose after the heat-shock treatment, was as thermotolerant as the wild-type strain, but also retained this thermotolerance for a longer time after downshift to 27°C (De Virgilio *et al.*, 1994). Insight into the contribution of both trehalose and hsps to the acquisition of thermotolerance was provided by another study. It could be shown that an *hsp104Δ* strain (unable to synthesize Hsp104 but able to accumulate trehalose) that was preconditioned at 40°C for 30 min was more sensitive to a challenging heat shock (>5min at 50.4°C) than the corresponding wild-type strain. However, when the preconditioning heat shock was extended to 60 min, the reduction in thermotolerance observed in the *hsp104Δ* strain almost disappeared. These findings indicated that the importance of Hsp104 for induced thermotolerance diminished upon prolongation of the preconditioning heat shock, coinciding with full induction of trehalose synthesis (De Virgilio *et al.*, 1991b). Therefore, the authors concluded that acquired thermotolerance is due to two different, independent mechanisms, namely protection from and repair of heat-stress damage. According to this conclusion, hsps would be responsible for the repair of heat-induced damage, while trehalose would be responsible for protection during heat stress (De Virgilio *et al.*, 1991b). Recently, a study investigating the thermotolerance of a *tps2Δ*, an *hsp104Δ*, and a *tps2Δ hsp104Δ* double mutant strain during stationary phase, confirmed the findings of De Virgilio *et al.* (1991b) and came to essentially the same conclusions, suggesting synergistic effects for trehalose and hsps in the acquisition of thermotolerance (Elliott *et al.*, 1996). Interestingly, another group has been able to demonstrate by *in vivo* NMR analysis that trehalose but not Hsp104 increased the membrane fluidity of *S. cerevisiae* cells during heat shock (Iwahashi *et al.*, 1995). Therefore, trehalose may indeed have the function of protecting membrane structures *in vivo*.

Based upon the findings that trehalose is a common carbohydrate in anhydrobiotic organisms, where its accumulation has been found to correlate with the survival of these organisms, it should be expected that trehalose has a similar effect in *S. cerevisiae*. Indeed, it has been convincingly shown that yeast cells that contain high amounts of trehalose, either in stationary phase or due to a conditioning heat shock at 40°C are able to survive desiccation (Gadd *et al.*, 1987; Hottiger *et al.*, 1987). The group of Panek has demonstrated that for cells to survive desiccation it is necessary that trehalose is present on the in- and the outside of the membrane. This is achieved by the activity of a trehalose carrier. Cells lacking this carrier were unable to acquire desiccation tolerance despite the fact that they accumulated trehalose intracellularly (Eleutherio *et al.*, 1993).

Freezing tolerance of yeast is of special interest for the baking industry, for the production of frozen dough (Panek, 1995; Attfield, 1997). It has been reported that yeast strains with high trehalose contents were especially suitable for this purpose (Oda *et al.*, 1986). Attfield *et al.* (1992) tested the ability of yeast strains that accumulated varying

amounts of trehalose and also found a correlation between trehalose content and the ability to survive freeze-thaw stresses.

The resistance of *S. cerevisiae* to osmotic stress during growth was related to the intracellular trehalose concentration, indicating that trehalose could act as an osmoprotectant (MacKenzie *et al.*, 1988). This function of trehalose has been demonstrated for other organisms like *E. coli* (for a review see Strøm and Kaasen, 1993) or *Euglena gracilis* (Takenaka *et al.*, 1997). However, in *S. cerevisiae* the role of trehalose in osmotic stress has been a matter of debate, since it has also been observed that osmotic stress induced glycerol synthesis and that strains not capable of accumulating glycerol were sensitive to hyperosmotic stress (Albertyn *et al.*, 1994). A recent publication resolved this seeming contradiction by showing that resistance to severe osmotic stress is strongly correlated with the accumulation of trehalose induced by these stress conditions. The accumulation of glycerol then seems to be more important under conditions of moderate osmotic stress, when trehalose synthesis was not induced (Hounsa *et al.*, 1998).

Some toxic chemicals are also known to induce trehalose accumulation in *S. cerevisiae*. Attfield (1987) found an increase of the trehalose content after treatment of yeast cells with ethanol, copper sulfate or hydrogen peroxide, but did not report whether this also increased the stress tolerance of these cells. The proteasome inhibitor MG132, a substance that inhibits protein breakdown at 30°C and induces many hsps, also caused trehalose accumulation. Interestingly, the treatment with the proteasome inhibitor conferred thermotolerance, as seen by the survival of treated cells of a subsequent challenging heat shock at 52°C. It could be shown that the thermotolerance did not correlate with the hsp but with the trehalose content, by testing the thermotolerance of the cells after removal of the inhibitor. Cells then rapidly became thermosensitive again, in accordance with the rapid degradation of trehalose, while hsps continued to accumulate (Lee and Goldberg, 1998).

Taken together, the results described are consistent with the suggestion that trehalose acts as a stress protectant in yeast during heat shock, desiccation, freezing stress and high osmolarity. The fact that trehalose also accumulates in response to a number of toxic chemicals can be taken as in indication for trehalose being part of a general stress response of yeast. The molecular mechanisms of the protective action of trehalose on cellular structures like proteins and membranes have been elucidated *in vitro*, and recent findings make it likely, that the same mechanisms are also effective *in vivo*.

The dual role of Tps1

Cloning of the *TPS1* gene, encoding the Tre6P synthase, revealed its identity with *CIF1*, a gene essential for growth on glucose (Gonzalez *et al.*, 1992; Bell *et al.*, 1992).

Moreover, the group of Thevelein demonstrated that two other mutations affecting the initial steps of glycolysis, namely *fdp1* and *byp1*, were also alleles of *TPS1/CIF1* (Van Aelst *et al.*, 1993). It was suggested that Tps1 may have an additional role besides trehalose synthesis in the regulation of glucose influx into glycolysis, and the *TPS1* gene was also named *GGS1* (for general glucose sensor) in this context (Van Aelst *et al.*, 1993). The effects of deletion of *TPS1* are highly pleiotropic. Besides the defect in Tre6P synthase activity and the resulting inability to accumulate trehalose, *tps1Δ* cells fail to grow on glucose or other rapidly fermentable carbon sources. In the presence of these sugars they hyperaccumulate sugar phosphates, especially fructose-1,6-bisphosphate, leading to a gradual depletion of the entire intracellular phosphate pool. *Tps1Δ* mutants are also deficient in glucose-induced inactivation of fructose-1,6-bisphosphatase and a number of other glucose-induced regulatory phenomena, they show defects in glycogen metabolism, and homozygous diploid *tps1* cells are unable to sporulate (Van Aelst *et al.*, 1993; Thevelein, 1994 and references cited therein). The growth defect of a mutant defective in the *TPS1* gene can be suppressed by either restricting sugar influx into glycolysis (by deletion of hexokinase or restriction of sugar uptake; Hohmann *et al.*, 1993; Blázquez and Gancedo, 1995) or by diverting accumulated glycolytic intermediates to the production of glycerol (Van Aelst *et al.*, 1991; Blázquez and Gancedo, 1995). All these observations are consistent with the interpretation that loss of *TPS1* leads to unrestricted influx of sugar into glycolysis and subsequent overflow of this pathway during the initiation of fermentation. Homologs of *TPS1* have also been cloned from other yeasts, *e.g. Kluyveromyces lactis* (*GGS1*; Luyten *et al.*, 1993) and *Schizosaccharomyces pombe* (*tps1*$^+$; Blázquez *et al.*, 1994). While deletion of the *GGS1* gene in *K. lactis* caused a similar phenotype as deletion of *TPS1* in *S. cerevisiae*, deletion of *TPS1* in *S. pombe* caused no growth defect in the presence of glucose. This suggests that the trehalose synthesis pathway may have a different role in *S. pombe*. The same is true for *E. coli otsA* mutants, they are also defective in trehalose synthesis but show no further glycolysis-related phenotype.

In order to explain the dual role of Tps1 as Tre6P synthase and as regulator of glycolytic flux in *S. cerevisiae*, three models have been proposed (reviewed in Thevelein and Hohmann, 1995). The first model suggests the existence of a 'General Glucose Sensor' (GGS), a complex consisting of a glucose carrier, a sugar kinase, and Tps1. According to this model, Tps1 has two distinct functions, one as a subunit of the Tre6P synthase/phosphatase complex and another one as a direct regulator of glucose transport and phosphorylation. How the latter regulatory function of glycolysis would be performed in detail, was still an unsolved issue in the initial proposal (Thevelein, 1992). The second model, also called the 'phosphate recovery hypothesis', suggests that trehalose synthesis would serve as a metabolic buffer system by recovering free phosphate that is required downstream in glycolysis for the glyceraldehyde-3-phosphate dehydrogenase reaction.

This model solves the phosphate sequestration problem not by proposing an influence on influx and/or phosphorylation of sugars but by diverting excessively synthesized sugar phosphates into trehalose synthesis. It was also predicted that the importance of trehalose synthesis in phosphate homeostasis would be restricted to the early transition period from the derepressed to the repressed state (Hohmann *et al.*, 1993). The third model is based upon the finding that in yeast hexokinase activity is strongly inhibited by Tre6P *in vitro* (K_i was 40 μM and 200 μM for hexokinase II and hexokinase I, respectively; Blázquez *et al.*, 1993). Accordingly, it was suggested that Tps1-generated Tre6P would cause a downregulation of sugar phosphorylation *in vivo* and thereby control glycolytic flux. The central question is which of these three models may most accurately describe the *in vivo* situation in *S. cerevisiae*. Currently, several independent labs are intensively studying this question and it may be anticipated that in the near future exciting new insights will emerge from these studies. In this context, the findings presented in the present thesis, together with the latest results of other labs, will be discussed in more detail in the General Discussion.

Stationary phase and nutrient signaling

In the section above on the role of trehalose in protection of *S. cerevisiae* cells, it has been described how trehalose is accumulated in yeast cells in response to a small set of environmental conditions, such as heat stress or nutrient limitation. During growth on glucose, trehalose synthesis is repressed or at least no net synthesis of trehalose occurs, whereas trehalose accumulation is induced coinciding with the depletion of glucose from the growth medium (Lillie and Pringle, 1980). It is therefore evident that some sort of control must be exerted upon trehalose accumulation, most probably by controlling the activities of the synthesizing and/or degrading enzyme(s). Another interesting observation is that trehalose accumulation coincides with times of growth arrest, indicating that the regulation of trehalose synthesis is, at least under conditions of nutrient limitation, connected in some way with the control of cell cycle progression. In this context it is important to consider that for yeast cells, but also for other organisms, it is of vital importance that a new round of cell division is only initiated in the presence of sufficient nutrients to complete the cycle. Therefore cells must be able to perceive the presence of a variety of different nutrients in their growth medium, in order to decide whether to commit themselves to a new cell cycle or whether to arrest growth and enter a quiescent stage, called G_0, outside of the cell cycle (de Winde *et al.*, 1997).

The elucidation of signal transduction pathways in general and nutrient signaling pathways especially, is one of the most rapidly evolving topics of biology. For microorganisms like yeast it is essential to be able to respond to changes in their environment in a

coordinated manner, also but not solely with respect to cell cycle progression. For instance, yeast cells inoculated into a rich, glucose-based medium and growing to stationary phase must cope with several drastic changes in their nutritional environment: Initially, they have to adapt to rapid fermentative growth on glucose. When glucose eventually becomes depleted or rather before this is the case, cells have to drastically change their metabolism to prepare for respiratory/gluconeogenic growth. Still later in the growth cycle, cells run out of vital nutrients, for instance carbon. In response to this starvation, yeasts like other microorganisms then cease to grow and enter a nonproliferating state, namely stationary phase or G_0 (Werner-Washburne *et al.*, 1993, see also below). The coordination of these different adaptations is achieved by different nutrient signaling pathways which involve nutrients (*e.g.* glucose or nitrogen) as primary messengers. For many regulatory effects induced by nutrients apparently at least partial metabolism of the nutrient is required. This observation already indicates one of the problems often encountered during studies of nutrient signaling: Since nutrients always also function as 'nutrients' *per se*, it is difficult to dissect this metabolic function from putative regulatory functions and to distinguish between primary and secondary effects (for a review on signal transduction see Thevelein, 1994).

Glucose is undoubtedly one of the main primary messengers in *S. cerevisiae*. Its addition to derepressed yeast cells is known to trigger a wide variety of regulatory phenomena. Several pathways are involved in the generation of these glucose induced regulatory phenomena (Thevelein and Hohmann, 1995). Of these, the most intensively studied pathways are the main glucose repression pathway and the Ras/cAMP pathway. The main function of the glucose repression pathway and its central enzyme Snf1 seems to be to keep cells in a glucose-repressed state while glucose is present in the medium. Thereby futile cycling between glycolysis and gluconeogenesis is prevented (Ronne, 1995). Interestingly, a functional Ras/cAMP pathway has been implicated in being both essential for coordinated cell division and growth, thereby also influencing the cell's decision to enter stationary phase, and for regulation of trehalose accumulation (Thevelein, 1992; Thevelein, 1994). The following paragraphs will therefore first provide a detailed description of stationary phase as a distinct developmental stage in the life cycle of yeast and then focus on the role of the Ras/cAMP pathway in the control of entry into and exit from stationary phase as well as of synthesis and degradation of trehalose.

Stationary phase

As has been described above, yeast cells are strongly dependent on their ability to react to variations in their nutrient supply. These naturally not only include qualitative changes in the composition of the growth medium (for instance fermentable versus non-fermentable carbon source), but also quantitative differences which may lead in the ex-

treme to starvation. As a consequence of their growth, which eventually results in depletion of the nutrient resources, and of the fact that yeast cells are not able to actively change their location in order to find more favorable growth conditions, it can be expected that starvation is probably a rather common situation in the life of a yeast cell. It is therefore not surprising that these organisms have found ways to cope with such unfavorable conditions. The mechanism by which yeast cells and also other microorganisms endure times of starvation is by entering a special state, usually referred to as stationary phase or G_0 in which they outlast the lack of nutrients and literally 'wait for better times'. It is known that yeast cells can survive prolonged periods of time in stationary phase without the addition of nutrients. Despite the fact that stationary phase seems to be a very common event for yeast cells, comparatively little is known about it. This may be due to a larger interest in growing yeast cells, since these are used for processes that are of great human interest, such as brewing, wine-making, or baking (for reviews on stationary phase see Werner-Washburne *et al.*, 1993; Werner-Washburne *et al.*, 1996; Fuge and Werner-Washburne, 1997).

It has proven to be difficult to exactly define stationary phase. With respect to a population of cells in a batch culture, one could define stationary phase as the phase when no further net increase in cell number is observed. However, such a definition would not characterize the exact physiological status of single cells in this culture. In the following paragraphs stationary phase will therefore be described as a physiologically distinct state outside of the mitotic cell cycle, which in this respect is equivalent to the G_0 phase of mammalian cells.

Yeast cells that are starved for an essential nutrient, usually carbon, arrest growth in G_1 and undergo a number of physiological, biochemical and morphological adaptations that set them apart from exponentially growing cells. In stationary phase, cells then appear as small, predominantly unbudded, and having large prominent vacuoles. Their cell wall is increased in thickness and therefore more resistant to cell-wall degrading enzymes. Cells also contain polyphosphates, have accumulated glycogen and trehalose, and they are more thermotolerant. Certain genes, including genes of the heat-shock family are induced, *e.g. SSA3*, *HSP26*, and *UBI4* (reviewed in Werner-Washburne *et al.*, 1993). However, these characteristics have also been found in cells that are in other growth phases and therefore they are a description but not a definition of stationary phase. For instance cell wall thickening and induction of thermotolerance has been connected with slow growth rather than with stationary phase (Elliott and Futcher, 1993; Gross and Watson, 1996). On the other hand it has also been suggested that cells growing with suboptimal nutrient supply may transiently enter stationary phase. Similarly, it has been proposed that even during normal exponential growth a certain percentage of the cells in a culture is in stationary phase. This hypothesis was based on the observation that unbudded cells in a growing culture could be divided into two groups based on their thermotol-

erance (Plesset *et al.*, 1987). The most reliable definition of stationary phase cells therefore still seems to be the ability of the cells to survive in the absence of nutrients (Werner-Washburne *et al.*, 1993).

As mentioned above, one ongoing discussion has been over the question whether yeast cells in stationary phase are really in a distinct state set apart from the cell cycle, referred to as G_0 or whether they are simply in a prolonged G_1 phase. This discussion mainly originated from the inability to distinguish between cells arrested in G_1, *e.g.* following a heat shock, and between cells arrested in growth because of slow nutrient limitation (see above). In mammalian cells, G_0 is brought about by the action of hormones and growth factors and it is characterized by the expression of a set of special genes, for instance the growth-arrest-specific (gas) genes. In yeast cells, no homologs of these genes have been identified so far (Fuge and Werner-Washburne, 1997). The finding that cyclin mRNA is absent from stationary yeast cells, however, speaks against the idea of a prolonged G_1 phase in which cells would not really arrest growth but continue to grow very, very slowly (Fuge and Werner-Washburne, 1997). Also, the identification of a novel mutant that is conditionally defective only for the resumption of proliferation from stationary phase has contributed to the understanding of stationary phase as an off-cycle developmental state (Drebot *et al.*, 1987). Stationary phase cells carrying the *gcs1* mutation are unable to resume growth if incubated at the restrictive temperature of 14°C. However, *gcs1* cells in exponential phase that were shifted to 14°C had no growth defect. The same was true for proliferating *gcs1* cells subjected to a heat shock at 37°C and then shifted to the restrictive temperature. These results not only define the role of *GCS1* as a true stationary phase gene but also demonstrate that heat shock does not cause cells to enter stationary phase, even though it causes cells to acquire some of the traits found in resting cells (Drebot *et al.*, 1990). Also, recently a new gene, *SNZ1* ('snooze'), has been identified that is expressed exclusively in stationary phase in *S. cerevisiae* and unlike other postdiauxic genes is not induced by heat shock. Interestingly, this gene was found to be a member of a highly conserved gene family present in all three phylogenetic domains: eucarya, bacteria, and archaea. Its function has not been clarified yet, but it is known that a Snz protein from *Bacillus subtilis* is modified during sporulation, indicating that Snz proteins are part of an ancient response to nutrient limitation and growth arrest (Braun *et al.*, 1996).

Another problem encountered when trying to define stationary phase specific characteristics is the fact that, probably due to the difficulties of defining stationary phase, researchers have used the expression 'stationary phase cell' in a rather broad sense. Since most studies are conducted with cells growing on rich or minimal glucose-based medium, often cells may be referred to as stationary that are in fact still in the postdiauxic phase, and therefore are actually still growing, albeit very slowly. Fuge and Werner-Washburne (1997) therefore have suggested to consider stationary phase as a developmental process

that is divided into three distinct phases: (i) Entry into stationary phase, which may still include a period of slow growth followed by a cell-cycle arrest. On glucose-based, rich medium this process would then include the diauxic shift and the postdiauxic phase. Most experiments dealing with stationary phase are performed on glucose-based rich medium so that little evidence is available upon which to base a decision whether cells being limited for different nutrients also enter stationary phase in a different way or even enter a different kind of stationary phase. In this context it has actually been suggested that only cells starved for glucose can enter a viable stationary phase. This suggestion was based on the observation that glucose alone is sufficient to stimulate growth in starved cells (Granot and Snyder, 1991). The authors argued that cells are always stimulated to grow as long as glucose is present and therefore lack of another vital nutrient would not be sufficient to overcome the stimulation of glucose. (ii) Maintenance of stationary phase, which is also referred to as quiescence. During this phase clearly the cell's priorities must be to retain viability for as long as possible. This apparently can last for very long times, since it has been reported that yeast cells have survived for 175 years (Prokesch, 1991). A strong decrease of the rates of protein synthesis has been observed in stationary phase that supports the consideration that certain areas of the metabolism are put on hold. Stationary phase cells only synthesize 0.3% of the amount of proteins synthesized in exponentially growing cells (Fuge *et al.*, 1994; Werner-Washburne *et al.*, 1996). Since the duration of stationary phase depends on the amount of time that starvation conditions prevail, cells have to be prepared to live off internal reserves for possibly a long time. It is known that stationary phase cells contain large amounts of compounds that may be regarded as potential energy sources, these include glycogen, trehalose and lipids (Lillie and Pringle, 1980). In the case of trehalose, its accumulation probably also promotes survival by protecting cellular structures in a more direct way (Wiemken, 1990). (iii) Reproliferation, the phase in which cells exit stationary phase to reenter the mitotic cycle. Having retained this ability to resume growth marks cells that have successfully proceeded through stationary phase. Cells resupplied with nutrients quickly loose their stationary phase characteristics, for instance they mobilize trehalose, their cell walls become susceptible to degrading enzymes, and small daughter cells left over from the last division before growth arrest increase in size before they divide (Johnston *et al.*, 1977; Thevelein, 1984b; Granot and Snyder, 1993). The *GCS1* gene has been identified as a gene that specifically affects the cell's ability to reenter mitosis from stationary phase (see above).

Taken together, the current state of knowledge about stationary phase in yeast indicates that this is indeed a distinct phase outside of the normal mitotic cell cycle. Future studies should certainly continue to try to elucidate further the molecular mechanisms underlying the complex processes in yeast cells during entry into, maintenance of viability in, and reproliferation from stationary phase. In this context it will be of great interest to identify genes and the proteins they code for that are distinctly important in stationary

phase. These could be for instance genes involved in regulating entry into stationary phase by slow nutrient depletion as opposed to growth arrest due to other forms of stress, like heat shock or rapid nutrient limitation.

The Ras/cAMP pathway

Several years ago it has been observed that addition of glucose to derepressed vegetative yeast cells or to ascospores triggers a rapid transient increase in the intracellular cAMP level (van der Plaat, 1974; Thevelein, 1984a). This cAMP signal has been implicated as an important metabolic trigger for the adaptation of the cells to fermentative growth. It is now known that the cAMP signal is transmitted by an elaborate signaling pathway, the Ras/cAMP pathway, resulting eventually in the activation of its key enzyme, the cAMP-dependent protein kinase (cAPK). The components of this pathway will be discussed in detail in the Introduction of Chapter III. Therefore this section will be focused on its involvement in signaling the nutritional status of the cell and in regulating trehalose synthesis.

The Ras/cAMP pathway is required for proper regulation of growth, cell cycle progression and development in response to nutritional conditions (Matsumoto *et al.*, 1983b; Thevelein, 1992; Hubler *et al.*, 1993). Its main function seems to be to signal the presence of glucose or another rapidly fermentable carbon source in the medium during growth and thereby to participate in the cell's decision to enter stationary phase. This is supported by the findings that mutants with hyperactivity or too little activity of cAPK display strongly pleiotropic phenotypes. Low cAPK activity, as conferred by mutations in the catalytic subunit of cAPK, leads to the induction of stationary phase adaptations before the depletion of nutrients, hyperactivity of cAPK prevents these adaptations even under starvation conditions. Total loss of cAPK is lethal, since it permanently locks cells in G_0 (for a review see Thevelein, 1994). cAPK activity may also play an important role in the general stress response of *S. cerevisiae*, possibly by phosphorylating proteins that modulate stress response in a negative way. In accordance with this model it has been suggested that stress conditions (*e.g.* heat shock, osmotic shock) may lead to a transient drop in cAPK activity, thereby triggering responses similar to those seen upon slow glucose depletion (Siderius and Mager, 1997).

Trehalose accumulation in *S. cerevisiae* seems to be at least partially controlled by the Ras/cAMP pathway. This is corroborated by the finding that a high cAMP level, and consequently high cAPK activity, in cells during growth on glucose coincides with the lack of trehalose accumulation, whereas during the diauxic shift trehalose accumulation is induced concomitantly with a sudden drop in the cAMP level. Also, mutations affecting the activity of the Ras/cAMP pathway also seriously affected trehalose metabolism. Mutations conferring constitutively high activity of cAPK resulted in low amounts of

trehalose even when cells were starved for nutrients, while mutations conferring constitutively low activity of cAPK, resulted in high amounts of trehalose even in the presence of nutrients (Toda *et al.*, 1985; Hottiger *et al.*, 1989). One explanation for these effects is based upon the observations that neutral trehalase is activated by cAMP-dependent phosphorylation *in vitro* and that neutral trehalase was more active in extracts from a strain with constitutively active cAPK than in extracts from a wild-type strain (Uno *et al.*, 1983; App and Holzer, 1989; see also section on neutral trehalase above). Consequently, trehalase would be inactive, thus allowing trehalose to accumulate, when cAPK is inactive, either due to low cAMP levels (*e.g.* at the diauxic shift) or because of inactivating mutations in the Ras/cAMP pathway. However, this model does not directly explain how inactivation of trehalase would be achieved and it also formally implies futile cycling of trehalose during growth on glucose in wild-type strains. It therefore seems reasonable to assume that the Ras/cAMP pathway may not only influence degradation but also synthesis of trehalose, in an antagonistic way with trehalase activity. This assumption is supported by the findings that exponentially growing cells (cAPK is active) have low Tre6P synthase activity *in vitro* and do not contain trehalose (Hottiger, 1988). In addition it has been found that the *in vitro* activity of Tre6P synthase was also affected in strains carrying mutations that either constitutively activated or inactivated cAPK. In accordance with an opposite regulation of trehalase and Tre6P synthase by cAPK, strains with an elevated level of cAPK activity displayed decreased Tre6P synthase activity *in vitro* (and failed to accumulate trehalose, see above) and strains with decreased cAPK activity displayed increased Tre6P synthase activity (and accumulated high levels of trehalose, see above; Panek *et al.*, 1987; François *et al.*, 1991). Panek *et al.* have claimed that the regulation mechanism by which cAPK affects Tre6P synthase activity is inactivation by phosphorylation (Panek *et al.*, 1987). However, the results of that particular study have been challenged and currently no evidence is available that would indicate such a control of trehalose synthesis by cAPK (Vandercammen *et al.*, 1989; see also Introduction of Chapter II). François *et al.* (1991) interpreted their results, showing an influence of cAPK activity on the activity of Tre6P synthase, as possibly involving transcriptional regulation of the gene encoding Tre6P synthase by cAMP. An influence of cAMP on the expression level of genes during the diauxic shift has been demonstrated. Addition of cAMP to wild-type cells growing on glucose prevented changes in gene expression normally occurring upon glucose depletion (Boy-Marcotte *et al.*, 1996). But it has not explicitly been shown that the expression of Tre6P synthase is affected by such a treatment. Therefore, at the moment, it is not clear how the Ras/cAMP pathway modulates Tre6P synthase activity.

Despite the efforts of many labs, a lot of open questions remain concerning the Ras/cAMP pathway. One problem is the fact that only very few of the targets of cAPK in vivo have been identified. Another difficulty is provided by the fact that several other nu-

trient signaling pathways are probably present in *S. cerevisiae* that functionally overlap or act in an antagonistic way to the Ras/cAMP pathway. Some of these have been identified, *e.g.* the Sch9 pathway, the Yak1 pathway, the Snf1 pathway, and the fermentable growth medium (FGM) induced pathway, but the relative contribution of most of these pathways to the responses of yeast to nutrients are still unclear (Hartley *et al.*, 1994; Thevelein, 1994; Ronne, 1995; Thevelein, 1996; de Winde *et al.*, 1997).

In order to ensure their survival, yeast cells are absolutely dependent upon their ability to react to changes in their environment in a fast and efficient manner. During evolution these organisms therefore have developed a number of fascinating strategies to overcome environmental limitations, be it the lack of nutrients or of water, or extreme changes in their ambient temperature. Accumulation of trehalose is one prerequisite for the cells' survival of unfavorable conditions. As described above, the synthesis of trehalose is not only an important part of the stress response but has also an essential role in the control of glycolytic flux. Elucidation of the regulatory mechanisms governing trehalose synthesis, one of the central goals of this thesis, will therefore undoubtedly prove to be of great value for the general understanding of both the stress response and glucose metabolism in microorganisms.

MATERIAL AND METHODS

Nomenclature

As described in this thesis, the *TAK1* gene was identified in a two-hybrid screen for interactors of Tps1, the Tre6P synthase, in a two-hybrid screen. Accordingly, it was named *TAK1* for **T**ps1 **a**ssociated protein **k**inase and its gene product Tak1. In the meantime, however, the same gene and its gene product have been described under the name *RIM15* and Rim15, respectively and shown to have an important function in the regulation of early meiotic events (Vidan and Mitchell, 1997). In agreement with the guidelines set forth by the Saccharomyces Genome Database, giving priority to the gene name usage that was first published, and for the sake of clarity, *RIM15* and Rim15 instead of *TAK1* and Tak1, will be used throughout this thesis.

Yeast strains and culture conditions

The yeast strains used in this study are listed in Table 1. Media were prepared according to Rose *et al.* (1990) and cells were grown at 27°C either in full YPD medium (1% [w/v] yeast extract, 2% [w/v] bacto-peptone, 2% [w/v] glucose) or in selective SD medium (0.67% [w/v] yeast nitrogen base without amino acids, 2% [w/v] glucose) supplemented with the appropriate auxotrophic requirements. For experiments with stationary phase cells cultures were grown on media containing only half the amount of carbon source to ensure this to be the limiting factor. For the determination of growth on different carbon sources these were added to the media as indicated in the figure or table legends. Cell numbers were counted using a hematocytometer. Dry weight was determined after filtering cells over a tared GF/C filter and drying the filter at 37°C over night. In order to study the consequences of nitrogen starvation, cells were pregrown on SD medium until mid-log phase ($2*10^7$ cells/ml) and then shifted to selective medium without nitrogen source (0.17% [w/v] yeast nitrogen base without amino acids and ammonium sulfate, 4% [w/v] glucose). The concentration of glucose in the media during growth was monitored with test strips (Diabur-Test, Boehringer Mannheim).

Bacterial strains and culture conditions

E. coli strain JMB9 ([r^-m^+] Δ*trpF*) (Sterner *et al.*, 1995) was used to rescue pJG4-5 based plasmids from strain EGY48. The transformed cells were plated directly onto

Table 1: *List of yeast strains*

Strain	Genotype		Source
RH144-3A	*MAT* α	*leu2 his4 ura3 bar1-1*	H. Riezman
AR3	*MAT* α	*leu2 his4 ura3 bar1-1 rim15::URA3*	This study
YEF473	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	Bi and Pringle (1996)
AR1	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rim15Δ::kanMX2/RIM15*	This study
AR1-1A	*MAT* **a**	*his3 leu2 lys2 trp1 ura3*	Segregant from AR1
AR1-1B	*MAT* α	*his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2*	Segregant from AR1
AR1-1C	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2*	Segregant from AR1
AR1-1D	*MAT* α	*his3 leu2 lys2 trp1 ura3*	Segregant from AR1
AR2	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rim15Δ::kanMX2/rim15Δ::kanMX2*	AR1-1B X AR1-1C
SP1	*MAT* **a**	*ade8 his3 leu2 trp1 urs3*	Toda *et al.* (1985)
T16-11A	*MAT* **a**	*his3 leu2 trp1 ura3 bcy1-1*	Toda *et al.* (1985)
PD6517	*MAT* α	*ade8 leu2 trp1 cdc35-10*	Becher dos Passos *et al.* (1992)
NB11	*MAT* α	*ade8 leu2 trp1 cdc35-10 rim15Δ::kanMX2*	This study
OL86	*MAT* α	*ade2 leu2 trp2 cdc25-5*	Becher dos Passos *et al.* (1992)
RS13-58A-1	*MAT* **a**	*ade8 his3 leu2 ura3 trp1 tpk1w tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*	Nikawa *et al.* (1987)
S7-7A x S7-5A	*MAT* **a**/α	*ade8/ade8 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 TPK1/tpk1::URA3 TPK2/tpk2::HIS3 TPK3/tpk3::TRP1*	Toda *et al.* (1985)
NB13	*MAT* **a**/α	*ade8/ade8 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 TPK1/tpk1::URA3 TPK2/tpk2::HIS3 TPK3/tpk3::TRP1 RIM15/rim15Δ::kanMX2*	This study

Strain	Genotype		Source
NB13-1D	*MAT* α	*ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1 rim15Δ::kanMX2*	segregant from NB13
NB13-14D	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 rim15Δ::kanMX2*	segregant from NB13
NB13-19C	*MAT* α	*ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 rim15Δ::kanMX2*	segregant from NB13
SGP406	*MAT* **a**	*ade8 his3 leu2 trp1* ura3 *tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2*	Garret and Broach (1989)
CDV80	*MAT* **a**/α	*TPK1/tpk1::URA3 tpk2::HIS3/ tpk2::HIS3 tpk3::TRP1/tpk3::TRP1 YAK1/yak1::LEU2 RIM15/rim15Δ::kanMX2*	NB13-1D x SPG406
CDV80-2C	*MAT* α	*ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1*	segregant from CDV80
CDV80-4B	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1*	segregant from CDV80
CDV80-4C	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1*	segregant from CDV80
CDV80-1D	*MAT* α	*ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2*	segregant from CDV80
CDV80-7B	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1 rim15Δ::kanMX2*	segregant from CDV80
CDV80-2B	*MAT* α	*ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1 rim15Δ::kanMX2*	segregant from CDV80
CDV80-15D	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2 rim15Δ::kanMX2*	segregant from CDV80
CDV80-33B	*MAT* α	*ade8 his3 leu2 trp1 ura3 ttpk2::HIS3 tpk3::TRP1 yak1::LEU2 rim15Δ::kanMX2*	segregant from CDV80
CDV80-2D	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2*	segregant from CDV80

Strain	Genotype		Source
CDV80-5A	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2*	segregant from CDV80
CDV80-15A	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 rim15Δ::kanMX2*	segregant from CDV80
CDV80-4A	*MAT* α	*ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2 rim15Δ::kanMX2*	segregant from CDV80
CDV80-8A	*MAT* **a**	*ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2 rim15Δ::kanMX2*	segregant from CDV80
CDV81	*MAT* **a**/α	*tpk1^{w}/tpk1::URA3 tpk2::HIS3/tpk2::HIS3 tpk3::TRP1/tpk3::TRP1 BCY1/bcy1::LEU2 RIM15/rim15Δ::kanMX2*	NB13-19C x RS13-58A-1
CDV81-3A	*MAT* α	*ade8 his3 leu2 ura3 trp1 tpk1^{w} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 rim15Δ::kanMX2*	segregant of CDV81
CDV81-11B	*MAT* α	*ade8 his3 leu2 ura3 trp1 tpk1^{w} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 rim15Δ::kanMX2*	segregant of CDV81
CDV81-16D	*MAT* **a**	*ade8 his3 leu2 ura3 trp1 tpk1^{w} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 rim15Δ::kanMX2*	segregant of CDV81
MH272-1D	*MAT* **a**	*leu2 his3 trp1 ura3 rme1*	M. Hall
EGY48	*MAT* α	*his3 trp1 ura3 LEU2::pLexAop6-LEU2*	Zervos *et al.* (1993)

Vogel-Bonner minimal plates (Davis *et al.*, 1980) supplemented with 0.2% (w/v) glucose, 0.5% (w/v) casamino acid hydrolysate, 0.01 mM $FeCl_3$ and 100 mg/l ampicillin. Other plasmid manipulations were performed in *E. coli* strain DH5α (Gibco BRL) using standard procedures (Sambrook *et al.*, 1989). *E. coli* media were prepared according to standard recipes (Sambrook *et al.* 1989).

Plasmid constructions

YCplac33-RIM15

Complementation of the *RIM15* deletion was achieved with a vector expressing *RIM15* under its own promoter, YCplac33-*RIM15*. The Expand Long Template PCR System (system 1) from Boehringer Mannheim was used according to the manual provided by the manufacturer to generate a 6.3 kb fragment (nucleotides -514 until 5811 of *RIM15*), including the entire ORF of *RIM15* and 514 nucleotides up- and 501 nucleotides downstream of the ORF. The primers, including a *Sma*I or a *Sal*I site, used in the PCR reaction were 17099 and 17100, respectively (see Table 2) and phage clone ATCC 70791 (from ATCC, Rockville, MD, USA; genomic DNA) was used as template. Due to the great length of *RIM15*, a cycle elongation program was used for the PCR reaction. In detail, the PCR program was as follows: denaturing of template DNA at 92°C for 2 min, followed by 10 cycles of 10 s at 92°C, 30 s at 60°C and 3 min at 68°C. After these 10 cycles further additional 17 cycles of 10 s at 92°C, 30 s at 66°C and 3 min at 68°C were performed. During these latter 17 cycles the elongation time was increased stepwise by 20 s per cycle, resulting in a final elongation time of 8 min 40 s. The program ended with 10 min at 68°C, after that the reaction was cooled to 4°C. The ends of the resulting PCR product were filled by Klenow treatment. To this means, 3 U of Klenow enzyme (Boehringer Mannheim) were added and the sample was incubated at 37°C for 1.5 h. After that it was phenolized and precipitated with ethanol. The fragment was then digested with *Sma*I and *Sal*I (Pharmacia) and ligated into the *Sal*I-*Sma*I sites of YCplac33 (Gietz and Sugino, 1988).

YCpIF2-derived plasmids

Galactose-inducible *GAL1* promoter-driven overexpression of full-length and truncated *RIM15* and of GST-tagged full-length and truncated *RIM15* was achieved with plasmids derived from YCpIF2 (Foreman and Davis, 1994). The full-length *RIM15* ORF was amplified using the Expand Long Template PCR System (Boehringer Mannheim) according to the manual. Primers 18412 and 18413 (Table 2) were included in the reaction mix with genomic DNA (phage clone ATCC 70791, see above) as template. With primer 18412 a *Sal*I immediately upstream of the start codon and with primer 18413 a *Not*I site

Table 2: *Primer list*

Number	Description	Orientation	Length	Sequence
13815	*RIM15*-S	F	26	5'-CTGATTCGCCGTCACAAGTTTGTCCCACATAAGTCG-3'
13816	*RIM15*-S	R	26	5'-CGTATTGGTAGCTGCGATAACGTCTGAAGATAATAG-3'
16421	*kanMX2* control	R	26	5'-GCATGGTTACTCACCACTGCGATCCC-3'
17099	*RIM15* cloning in YCplac33	R	38	5'-CTTCCAATTGTTAAGTCCCGGGAGAACTATTCTTCCAG-3'
17100	*RIM15* cloning in YCplac33	F	39	5'-CAAAACCACGTTCATCAGTCGACACGATGTATATTTGCG-3'
17921	*RIM15* deletion	F	62	5'-CTCTTGCCTCATTTGATAGAATAGATAAGCCCAGTAGAGGAAGCAG CTGAAGCTTCGTACGC-3'
17922	*RIM15* deletion	R	62	5'-CCGTTTTAATGAACGGGGGAAAATCCATGATTATTCTCAGGCATAG GCCACTAGTGGATCTG-3'
18060	*RIM15* deletion control	F	29	5'-CCTCTTAACCACTCCTGGAAGGCCTGCTG-3'
18061	*RIM15* deletion control	R	29	5'-GCTAGTCCAGGATCCTGATCATCATTGAG-3'
18412	*RIM15* cloning in YCpIF2	R	45	5'-CCGTTTTAATGAGCGGCCGCAAATCCATGATTATTCTCAGATTTG-3'
18413	*RIM15* cloning in YCpIF2	F	42	5'-CAGTAGAGGAGTCGACATGTTCAATAGAAGTAACACCGCAGG-3'
21486	*RIM15-P1* cloning in YCpIF2	R	42	5'-GGGACAAAAGAAGCGGCCGCATCGTAAACATGATCCCAATCC-3'
21488	*RIM15-P2* cloning in YCpIF2	F	41	5'-GCCAGAACAGATAGTGTCGACAATGCAATGTTAACTTCACC-3'

Number	Description	Orientation	Length	Sequence
21765	*TPK1* cloning in pEG202	F	49	5'-GAAAGAATCTTTTTTGAATTCATGTCGACTGAAGAACAAAATGGAG GTG-3'
21766	*TPK1* cloning in pEG202	R	40	5'-GTTCCATCTCTCGAGTAACTATACAGGGGTTGAAATTTCG-3'
22129	*GST* cloning in YCpIF2	F	36	5'-CACACAGGAAACAGTCGACATGTCCCCTATACTAGG-3'
22130	*GST* cloning in YCpIF2	R	34	5'-CGTCAGTCAGTGTCGACGAATTCCCGGGGA-3'
	ADH2 probe	F	27	5'-ATGTCTATTCCAGAAACTCAAAAAGCC-3'
	ADH2 probe	R	26	5'-TTATTTAGAAGTGTCAACAACGTATC-3'
	HSP12 probe	F	27	5'-ATGTCTGACGCAGGTAGAAAAGGATTC-3'
	HSP12 probe	R	27	5'-TTACTTCTTGGTTGGGTCTTCTTCACC-3'
	HSP26 probe	F	30	5'-ATGTCATTTAACAGTCCATTTTTTGATTTC-3'
	HSP26 probe	R	30	5'-TTAGTTACCCCACGATTCTTGAGAACAAAC-3'
	SSA3 probe	F	18	5'-ATGTCTAGAGCAGTTGGT-3'
	SSA3 probe	R	18	5'-ATCAACCTCTTCCACTGT-3'
	SSB1 probe	F	26	5'-ATGGCTGAAGGTGTTTTCCAAGGTGC-3'
	SSB1 probe	R	27	5'-TTAACGAGAAGACATGGCCTTGGTGAC-3'
	UBI4 probe	F	18	5'-ATGCAGATTTTCGTCAAG-3'
	UBI4 probe	R	18	5'-GTTACCACCCCTCAACCT-3'

Number	Description	Orientation	Length	Sequence
K823Y-F	Y for K substitution in Rim15, position 823	F	50	5'-CAGGAGATTATTTTGCTATATACGTTCTAAGGAAATCAGATATGATTG-3'
K823Y-R	Y for K substitution in Rim15, position 823	R	50	5'-GGCAATCATATCTGATTTCCTTAGAACGTATATAGCAAAATAATCTCCTG-3'
S709A-F	A for S substitution in Rim15, position 709	F	37	5'-CCCCAAGGCGTGGTGCACCATCATTTGGTAATCTCGC-3'
S709A-R	A for S substitution in Rim15, position 709	R	37	5'-GCGAGATTACCAAATGATGGTGCACCACGCCTTGGGG-3'
S1094A-F	A for S substitution in Rim15, position 1094	F	45	5'-GCTAATAATATTATGAGAAGGAAAGCACTCACTGAGAATAAATCC-3'
S1094A-R	A for S substitution in Rim15, position 1094	R	45	5'-GGATTTATTCTCAGTGAGTGCTTTCCTTCTCATAATATTATTAGC-3'
S1416A-F	A for S substitution in Rim15, position 1416	F	41	5'-GAGGGATCGCAGATCAGCTAAATTGAATGATTCACAAACGG-3'
S1416A-R	A for S substitution in Rim15, position 1416	R	41	5'-CCGTTTGTGAATCATTCAATTTAGCTGATCTGCGATCCCTC-3'
S1463A-F	A for S substitution in Rim15, position 1463	F	39	5'-GGGGTTCACAGAAGAACCGCTTCTGCGTCACTAATGGGG-3'

Number	Description	Orientation	Length	Sequence
S1463A-R	A for S substitution in Rim15, position 1463	R	39	5'-CCCCATTAGTGACGCAGAAGCGGTTCTTCTGTGAACCCC-3'
S1661A-F	A for S substitution in Rim15, position 1661	F	41	5'-CTTTAAGAAACAGGAGGCGTGCTGGCCGAAAGAGCTCGAGC-3'
S1661A-R	A for S substitution in Rim15, position 1661	R	41	5'-GCTCGAGCTCTTTCGGCCAGCACGCCTCCTGTTTCTTAAAG-3'

All oligonucleotides used for PCR amplifications (see text) are listed in this table. Primer numbers where mentioned correspond to the identification numbers given by the FMI oligonucleotide synthesis lab. Orientation refers to the primer being either forward (F) or reverse (R). The length of the primers is given as number of nucleotides. Underlined sequences of primers 17921 and 17922 denote that part of the primer being complementary to the *kanMX2* module (see Material and Methods and Figure 2A).

86 bp downstream of the stop codon was introduced. The resulting PCR-product was cut with *Sal*I and *Not*I and ligated into the corresponding restriction sites of YCpIF2 to yield YCpIF2-*RIM15*. Partial versions of *RIM15* were generated by PCR, using primers 21488 with 21486 or 21488 with 18412 (Table 2), yielding a 1.5 kb or a 3.12 kb fragment encoding amino acids 761-1266 (*RIM15-P1*) or 762-1770 (*RIM15-P2*), respectively. *Sal*I and *Not*I restriction sites were introduced with the primers and the PCR products cloned at the *Sal*I-*Not*I sites of YCpIF2 (YCpIF2-*RIM15-P1* or YCpIF2-*GST-RIM15-P2*).

For the expression of *GST*-tagged *RIM15, RIM15-P1, RIM15-P2,* or *GST* alone as control, a PCR-generated *Sal*I-*Sal*I fragment containing the 672 nucleotides downstream of and including the *GST* start codon was amplified by PCR using primers 22129 and 22130 and pGEX1 (Smith and Johnson, 1988) as template and subsequently cloned at the *Sal*I sites of YCpIF2-*RIM15,* YCpIF2-*RIM15-P1,* YCpIF2-*RIM15-P2,* or YCpIF2. The resulting plasmids were called YCpIF2-*GST-RIM15,* YCpIF2-*GST-RIM15-P1,* YCpIF2-*GST-RIM15-P2,* and YCpIF2-*GST*, respectively. By sequencing, it was confirmed that the *GST*-tag was in-frame with the fused *RIM15* gene.

Plasmids YCpIF2-*GST-RIM15*^{K823Y} and YCpIF2-*GST-RIM15*$^{S709A/S1094A/S1416A/S1463A/S1661A}$ were constructed with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) using appropriate primers (Table 2) that introduced the corresponding mutations and YCpIF2-*GST-RIM15* as template. The principle of this kit is explained in Figure 1. All mutations introduced were confirmed by subsequent sequencing.

Constitutive *ADH1*-driven overexpression of *RIM15* was achieved with another YCpIF2-derived plasmid. To this means, a PCR-generated *Apa*I-*Sal*I fragment containing the 854 nucleotides upstream of and including the *ADH1* start codon was used to replace the *GAL1* promoter-containing *Apa*I-*Sal*I fragments of YCpIF2 and YCpIF2-*RIM15*.

pEG202-TPK1

The full-length ORF of *TPK1* was amplified by PCR with primers 21765 and 21766 using genomic DNA as template, introducing *Eco*RI and *Xho*I restriction sites with the primers. The resulting fragment was then cut and ligated into vector pEG202 at the corresponding *Eco*RI and *Xho*I sites.

pWB204Δ-236

The *SSA3-lacZ* fusion plasmid pWB204Δ-236, containing part of the *SSA3* promoter fused to *lacZ*, was a gift of Elizabeth Craig (University of Wisconsin, Madison, USA). It was used to monitor the activation of targets downstream of the cAMP-dependent protein kinase (cAPK) by determining the β-galactosidase activity. A detailed description of the plasmid has been published elsewhere (Boorstein and Craig, 1990a).

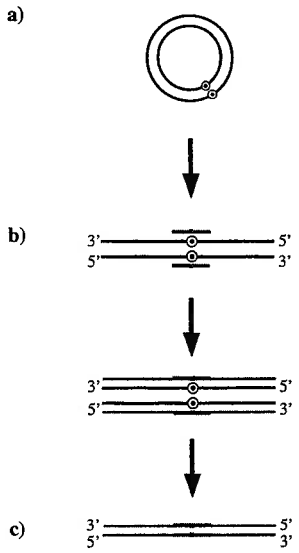

Figure 1: Site directed mutagenesis of *RIM15* using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). With this method it is possible to introduce desired mutations into a gene of interest, using a simple temperature cycling program. (a) A plasmid containing the gene of interest, in this case *RIM15* in vector YCpIF2, is used as template for the mutagenesis. (b) In a PCR reaction two oligonucleotide primers (K823Y-F and K823Y-R; S709A-F and S709A-R; S1094A-F and S1094A-R; S1416A-F and S1416A-R; S1463A-F and S1463A-R; S1661A-F and S1661A-R; see Table 2), containing the desired mutation, are extended by means of Pfu DNA polymerase. Pfu DNA polymerase is an enzyme with especially high fidelity. The reaction yields a mutated plasmid containing staggered nicks. (c) The temperature cycling is followed by treatment of the PCR-product with the *Dpn*I endonuclease. This enzyme is specific for methylated and hemimethylated DNA. Since DNA isolated from almost all *E. coli* strains is methylated, whereas the plasmid amplified by the temperature cycling program is not, *Dpn*I digests specifically the template DNA. The remaining nicked vector DNA containing the desired mutation in the target gene (*e.g. RIM15*^{K823Y}) is then transformed into a special strain of supercompetent *E. coli* (Epicurian Coli® XL-Blue). The nicks in the mutated plasmid are repaired by the cells after transformation.

pGEX3X-TPS1

The *TPS1* coding sequence was cut out with *Bam*HI from vector pEG202-*TPS1* (see Experimental procedures in Chapter I of this thesis for a description of this vector) and cloned at the *Bam*HI site of pGEX3X (Smith and Johnson, 1988). The resulting vector pGEX3X-*TPS1* was used to express GST-tagged *TPS1* in *E. coli* for subsequent purification of GST-Tps1.

pMAL-TPS1

The *TPS1*-coding sequence was cut out from vector pEG202-*TPS1* (see pGEX3X-*TPS1*) by digesting with *Eco*RI and *Pst*I (digest with *Eco*RI was only partial because of an internal *Eco*RI site in *TPS1*). The excised fragment was then cloned at the corresponding restriction sites of pMAL-c2 (New England Biolabs) to yield pMAL-*TPS1*. This vector allows expression of maltose-binding protein- (MBP) fused Tps1 in *E. coli.*

pMAL-TPK1

Analogous to the cloning of pMAL-*TPS1*, the *TPK1* gene was cut out from vector pEG202-*TPK1* with *Eco*RI and *Pst*I and cloned into pMAL-c2. Vector pMAL-*TPK1* allows expression of MBP-fused Tpk1 in *E. coli.*

Preparation of genomic or plasmid DNA from yeast

Genomic or plasmid DNA was prepared according to the protocol of Hoffman and Winston (1987). 5 ml of a saturated yeast culture were harvested and the cells washed once with sterile water. The pelleted cells were then resuspended in 0.2 ml of miniprep solution (2% [v/v] Triton X-100, 1% [w/v] SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 0.2 ml phenol-chloroform-isoamyl alcohol (25:24:1), and 0.3 g of glass beads were added. This mixture was vortexed continuously for 3-4 min (5 min for plasmid DNA). Afterwards 0.2 ml TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) were added, mixed briefly and the samples were centrifuged in a microfuge for 5 min at full speed. The aqueous layer was transformed to a new tube, 1 ml ethanol was added and mixed by inversion. The precipitated DNA was then pelleted by centrifugation for 2 min at full speed in a microfuge and washed once with 70% (v/v) ethanol. The pellets were dried briefly and resuspended in an adequate volume of TE (20-100 µl) and subsequently used as template for PCR reactions or in Southern blots.

Yeast transformations

Yeast transformations were performed using a modification of the Li^+-ion method (Gietz *et al.*, 1992). Selections of transformants were then done on appropriate amino acid drop-out media. For selecting transformants with the *kanMX2* module, cells were first grown for two days on YPD and then replica-plated onto YPD containing 1 mg/ml geneticin (G418, USBiological). After 2-3 days large colonies were picked and reselected on YPD with 0.2 mg/ml geneticin.

Two-hybrid screens with Tps1 and Tps2

Screens for proteins interacting with Tps1 (Tre6P synthase) and Tps2 (Tre6P phosphatase), were performed by two-hybrid analysis (Fields and Sternglanz, 1994), using the LexA system described in detail elsewhere (Gyuris *et al.*, 1993). All components of the two-hybrid system have been kindly provided by Roger Brent (Harvard University, Cambridge, MA, USA). *TPS1* and *TPS2* were fused to the LexA DNA-binding domain (DBD) of the "bait" vector pEG202 as described in the Experimental Procedures in Chapter I. Expression from this vector is under the control of an *ADH1* promoter. Strain EGY48 (Table 1), containing the *LexAop-lacZ* reporter plasmid pSH18-34 (Gyuris *et al.*, 1993) was used as host for all interaction experiments. This strain was either transformed with pEG202, pEG202-*TPS1* (for the *TPS1*-screen), or pEG202-*TPS2* (for the *TPS2*-screen) and with a yeast interaction library in the vector pJG4-5 (a gift from Paul Watt, Harvard University, Cambridge, MA, USA). Hybrid proteins consisting of the putative interactor fused to the LexA activation domain (AD), were expressed under the control of the *GAL1* promoter of the library plasmid. The transformation efficiency was determined and an appropriate number of transformations was performed to saturate the screen. Cells were plated on SD agar containing 2% (w/v) galactose and 1% (w/v) raffinose (no amino acids). Under these conditions only those clones expressing proteins interacting with DBD-Tps1 or DBD-Tps2, respectively, were expected to grow. Positive clones growing on SD with galactose without leucine were picked and streaked on (i) SD with 2% (w/v) galactose, (ii) SD with 2% (w/v) glucose, (iii) SD with 2% (w/v) galactose, 1% (w/v) raffinose and X-gal (40 µg/ml), and (iv) SD with 2% (w/v) glucose and X-gal (40 µg/ml). True positives should grow on (i), not grow on (ii), form blue colonies on (iii) and stay white on (iv). Those clones passing this test were further analyzed. Plasmid DNA was prepared from these positives and transformed into *E. coli* strain JMB9 (see above) to rescue the library plasmid. DNA from *E. coli* was isolated and subjected to restriction analysis with both *Eco*RI and *Hind*III, *Xho*I, or *Sau*3A. Clones showing the same restriction pattern were considered to contain the same library fragment

and assigned to one class of interactors. One insert of each class of positive interactors was then sequenced. If possible, the library fragment was identified by sequence comparison with the EMBL data base. Those plasmids containing sequences coding for transcription factors or other known "false positives" (see Chapter II, Results) were discarded. All rescued library plasmids were retransformed into strain EGY48 containing the reporter plasmid and pEG202 or a pEG202-derived plasmid (pEG202-*TPS1* or pEG202-*TPS2* as "baits" or pEG202-*MSB2* as negative control; Simon *et al.*, 1995; Reinders *et al.*, 1997). β-Galactosidase activities were then assayed on galactose media as described below. At this point, all library fragments yielding high β-galactosidase activity if cotransformed with the pEG202-*TPS1* or pEG202-*TPS2* but not with pEG202 or pEG202-*MSB2* control were considered positives.

Cloning of *RIM15*

A genomic yeast DNA library prepared in pSEY8 (a gift of Michael Hall, Biocenter, Basel) was screened by colony hybridization, utilizing the radiolabeled 0.52 kb insert of an *Eco*RI digested interaction library plasmid (clone #6 of *TPS1* two-hybrid screen, coding for amino acids 761-1051 of the *RIM15* gene) as described in Sambrook *et al.* (1989). Positive clones were confirmed by restriction analysis. The longest clone, pSEY8-5 (about 9 kb in length) was then chosen for sequencing.

DNA sequencing and sequence analysis

Sequences were either obtained using the dideoxy chain termination method (Sanger *et al.*, 1977) with the Sequenase kit, version 1.0 (USB Corp., Ohio) and [^{35}S] dATP (Amersham) according to the manual or using a cycle sequencing program and an automated sequencer (ABI 301, Perkin Elmer). For this, 1 µl DNA (0.5 µg) was mixed with 1 µl primer (0.5 µM), 4 µl reaction mix (Perkin Elmer) and 4 µl water. Cycle sequencing was performed in a Techne GENIUS PCR machine with a heated lid. The program used was: 27 cycles of 30 s at 96°C, 15 s at 50°C and 4 min at 60°C. After the completion of the program, 10 µl of water were added to the reaction, the DNA was precipitated with sodium acetate and ethanol and the pellet washed twice with ice-cold 70% (v/v) ethanol. The pellet was dried and 25 µl of template suppressing reagent (TSR, Perkin Elmer) were added. After 2 min incubation at 95°C the samples were ready for sequencing.

Oligonucleotides were synthesized with an Applied Biosystem DNA Synthesizer. Sequences were analyzed by means of the GCG package (Devereux *et al.*, 1984).

Gene disruption of *RIM15*

Gene disruption was carried out by the one step method of Rothstein (1983). A 1.7 kb *Nsi*I-*Xba*I fragment, covering one third of the coding region of the *RIM15* gene (Figure 2) was generated by PCR using genomic DNA of strain RH144-3A as template. This fragment was cloned into the multi cloning site of pCR™ (Invitrogen). A 1.4 kb *Bgl*II-*Bgl*II fragment containing the *URA3* gene was then excised from pVT102-U (Vernet *et al.*, 1987) and cloned into the *Bam*HI site of the 1.7 kb *Nsi*I-*Xba*I fragment of *RIM15*. The disrupted sequence was excised with *Cla*I-*Cla*I to yield a 2.3 kb linear fragment that was used for transformation into the wild-type strain RH144-3A, resulting in the gene disruption mutant AR3. The correct gene disruption was confirmed by Southern blot analysis (see below).

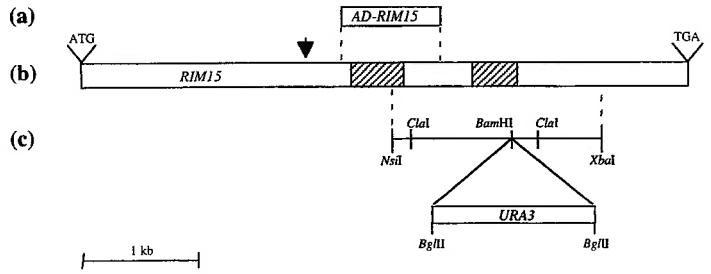

Figure 2: Summary of *RIM15* cloning and gene disruption strategy. (a) Size and localization of the LexA clone (*AD-RIM15*) which was identified during the two-hybrid screen for Tps1 interactors. (b) Structure of the 5.3 kb *RIM15* ORF. The kinase domains are indicated as shaded areas. Start (ATG) and stop (TGA) are marked. Kinase domains VII and VIII are separated by an unusual 0.63 kb stretch of DNA sequence. The arrow points to the start originally identified from the pSEY8 library clone #5 (see text). (c) The *URA3* marker (1.4 kb) was inserted into the *Bam*HI site of the PCR-generated *Nsi*I-*Xba*I fragment of *RIM15*, thereby disrupting the gene. This fragment was then cut with *Cla*I and the resulting 2.3 kb disruption construct used for transformation.

Construction of *rim15Δ* strains

Deletion of *RIM15* was achieved by the PCR-based one step method using the *kanMX2* module coding for geneticin (G-418) resistance as marker (Wach *et al.*, 1994; Figure 3A). First, DNA fragments with *RIM15* homologous DNA ends were generated by PCR: A 1.56 kb long PCR fragment was synthesized by using pFA6-*kanMX2* (provided by A. Wach, Basel) as template and two primers (17921 and 17922) with 19 or

22 nucleotide homology to the pFAG-*kanMX2* multiple cloning site (Table 2). In addition, these primers had 43 or 40 nucleotide extensions which were complementary to the regions immediately up- or downstream to the open reading frame (ORF) of *RIM15*. In 100 µl reaction volume 5 U of *Taq* DNA polymerase (Pharmacia), 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 0.4 µM of each primer, and 10 ng of template DNA were used. The whole reaction was overlaid with 3 drops of mineral oil (Perkin Elmer). The reaction conditions were: 2 min at 92°C to denature the template DNA, followed by 28 cycles of 30 s at 92°C, 30 s at 55°C, and 90 s at 72°C. Finally, the reaction mix was heated to 72°C for 4 min and then cooled to 10°C. 2µl of the PCR product were analyzed by agarose gel electrophoresis. Approximately 1 µg of the PCR-generated linear *RIM15*-deletion construct was directly used to transform the diploid wild-type strain YEF473. A heterozygous diploid transformant (AR1) was identified by its geneticin-resistance. Correct integration of the marker at the target locus was confirmed by Southern blot (see below) and/or by PCR as described below. Deletion of *RIM15* in other strain backgrounds (see Table 1) was achieved with the same PCR-based strategy.

Correct integration of the PCR-generated *kanMX2* module at the genomic *RIM15* locus was verified by PCR using genomic DNA of the transformants and three primers per reaction tube (Figure 3B): one reverse primer in the *kanMX2* module (16421), one reverse primer in the *RIM15* ORF (18061), and one forward primer outside of the *RIM15* ORF (18060). PCR reactions were done in a 50 µl volume, containing 2.5 U *Taq* polymerase (Pharmacia), 10 mM Tris-HCl (pH 9.0), 1.5 - 5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 0.4 µM of each primer, and approximately 1 µg of template genomic DNA. The mixture was overlaid with mineral oil (Perkin Elmer) and used with the following PCR conditions: denaturing of template DNA at 92°C for 2 min, followed by 30 cycles of 1 min at 92°C, 30 s at 63°C, and 1 min at 72°C, finally 5 min incubation at 72°C and eventually cooling to 10°C. The PCR products were run on agarose gels to check the resulting fragment size and to determine whether integration at the *RIM15* locus had occurred. Successful integration of the marker at the *RIM15* locus yielded a 950 bp PCR-fragment, in the case of integration at another locus the PCR product was 1.37 kb (see Figure 3B).

Southern blot analysis

Southern blot analysis to check the disruption or deletion of *RIM15* was performed essentially as described (Southern, 1975; Sambrook *et al.*, 1989). In short, 10 µg of genomic DNA of each strain were digested with *Cla*I (*RIM15* disruption) or with *Xho*I (*RIM15* deletion) overnight at 37°C. The DNA was precipitated with ethanol and sodium

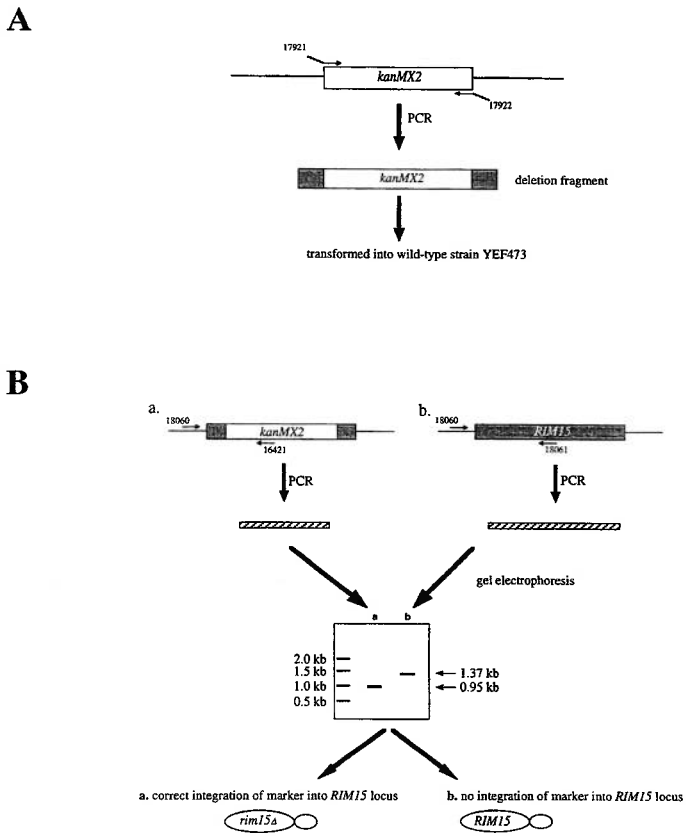

Figure 3: A. Construction of *rim15Δ* strains (Wach *et al.*, 1994). A PCR reaction was performed with the plasmid pFAG-*kanMX2* as template using two primers (17821, 17922) with homology to the *kanMX2* module as well as to the immediate up- and downstream region of the *RIM15* ORF (printed in grey). The resulting 1.56 kb DNA fragment therefore carried the *kanMX2* gene embedded in *RIM15* ORF flanking sequence (grey shaded areas). This fragment was transformed into wild type strain YEF473 (*RIM15/RIM15*) where it integrated by homologous recombination into one *RIM15* locus, resulting in the heterozygous strain AR1 (*RIM15/rim15Δ*). **B.** Confirmation of *rim15Δ* by PCR. The correct integration of the *kanMX2* marker at the *RIM15* locus and therefore the deletion of *RIM15* was checked by PCR. For the PCR reaction three primers were used, one reverse primer in the *kanMX2* module (16421), one reverse primer in the *RIM15* ORF (18061), and one forward primer in the *RIM15* promoter (18060). Genomic DNA of the transformed yeast strains was used as template. The length of the PCR product depends upon the combination of primers that can bind to the DNA: (a) *kanMX2* has integrated correctly, primers 18060 and 16421 bind and the result ing product is 0.95 kb long; (b) *kanMX2* has not integrated correctly, *RIM15* has not been deleted, primers 18060 and 18061 bind and the resulting product is 1.37 kb long.

acetate, the pellet washed with ice-cold 70% (v/v) ethanol, and taken up in 10-20 µl TE. Samples were mixed with loading buffer containing RNase and run on a 0.8% (w/v) agarose gel at 50 V for 4 h. The gel was stained in ethidium bromide solution (1 µg/ml) for 3 min to visualize bands. Afterwards the DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 40 min with gentle agitation. The gel was then soaked for 15 min in 0.25 M HCl, rinsed briefly with water and neutralized by soaking in 0.5 M Tris, 1.5 M NaCl for 40 min, all with gentle agitation. Subsequently, the DNA was blotted to nitrocellulose membrane (BA83, Schleicher & Schüll) by overnight capillary transfer, using 10x SSC (1.5 M NaCl, 170 mM sodium citrate, pH 7.0) as transfer buffer. The membrane was washed in 2x SSC, sandwiched between 3MM paper (Whatman), and baked in a vacuum oven at 80°C for 2 h to fix the DNA to the nitrocellulose. For the following prehybridization, the membrane was incubated in 10 ml hybridization solution (5x Denhardt's [1 mg/ml Ficoll type 400, 1 mg/ml polyvinylpyrrolidone, 1 mg/ml BSA], 2x SSC, 100 µg/ml salmon sperm DNA) in a hybridization oven (Hybaid) at 65°C for 2 h. In the meantime, the probe was labeled with ^{32}P using the Random Primed DNA Labeling Kit (Boehringer Mannheim). For the control of *RIM15* disruption and deletion, the internal 0.9 kb *Cla*I-*Cla*I fragment (amino acids 966-1336) and a 0.52 kb PCR-generated fragment (primers 13815 and 13816, *RIM15-S*, see Table 3) were used as probes, respectively. Hybridization was performed in a volume of 10 ml hybridization solution at 65°C overnight. Afterwards the membrane was washed three times with 2x SSC, 0.1% (v/v) SDS at 65°C. The membrane was wrapped in plastic wrap and exposed to X-ray film.

Sporulation and tetrad analysis

Diploid cells were grown for two days on full medium (YPD), harvested by centrifugation, washed twice with sterile water and then resuspended in the same volume of sporulation medium (1% [w/v] K-acetate, supplemented with 10 µg/ml of the required amino acids). After 3-6 days asci were dissected. For this purpose 0.4 ml of the sporulated yeast culture was pelleted, washed with sterile water and the pellet resuspended in 180 µl of sterile water. 20 µl of glusulase (NEE-154, Dupont) were added and the suspension was incubated at room temperature for 10-20 min, depending on the strain used. Afterwards 1 ml of water was added to the suspension and the mixture was placed on ice for at least 2 h before dissecting the asci.

The needle for the dissection of asci was constructed by drawing out a glass microcapillary with a gas flame. The needle was checked under a binocular to make sure that it had a flat end and then mounted into the micromanipulator. Asci were dissected at a magnification of 150x as described (Guthrie and Fink, 1991). In short, asci with four

spores were broken with the needle tip and the individual spores placed at distinct positions on very thin agar plates. From the spores colonies formed typically after 2-3 days of incubation at room temperature. Differences in colony size and growth speed were protocolled. Segregation of the different markers was tested on the appropriate selective media.

RNA isolation from yeast

RNA was prepared according to the protocol of Piper (1994). 30-50 ml of log-phase or 10-25 ml of stationary phase cells were harvested, chilled rapidly by adding ice cold DEPC-treated sterile water and pelleted. The cells were then washed once more with ice-cold DEPC-treated sterile water, pelleted and either frozen at -20°C for later processing or immediately extracted. 1-2 g of glass beads plus 2 ml RNA extraction buffer (20 mM Tris-HCl, pH 8.5, 10 mM Na_2-EDTA, 1% [w/v] SDS) and 2 ml phenol (pH 8.0) were added to the pellets and the mixture vortexed continuously for 5 min at room temperature. After centrifugation for 5 min at 3'500 rpm (IEC Centra GP8R) the upper aqueous phase was transferred to a new tube containing phenol-chloroform in an equal volume. The suspension was vortexed for 1 min, centrifuged for 5 min at 3'500 rpm and the upper phase transferred to a fresh tube containing an equal volume of chloroform. Again the suspension was vortexed for 1 min, centrifuged for 2 min at 3'500 rpm and the upper phase transferred to corex tubes. 6 M ammonium acetate to a final concentration of 1 M plus 2 volumes of ice cold ethanol were added and the tubes placed at -20°C for at least 20 min. The RNA was pelleted by centrifugation at 7'500 *g* for 15 min at 4°C, the supernatant poured off and the tubes drained on paper tissue. The pellets were resuspended in 1 ml TE and the RNA reprecipitated by adding 3 M sodium acetate to a final concentration of 0.2 M plus 2.5 volumes of ice-cold ethanol. After centrifugation at 7'500 *g* for 15 min at 4°C the pellets were washed once with ice-cold 70% (v/v) ethanol and then dried at room temperature. Eventually the RNA was suspended in an appropriate amount of TE (100-500 µl) and the concentration determined by reading the OD260/280 in a spectrophotometer. On a pre-gel, RNA integrity and equal loading were confirmed.

Northern blot analysis

For Northern blot analysis (Sambrook *et al.*, 1989) samples were prepared by resuspending 10 µg of total RNA in a final volume of 10 µl sample buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, pH 8.0, 2.2 M formaldehyde, 50% [v/v] formamide), and followed by incubation at 55°C for 10 min. Samples were mixed with loading buffer and 1 µl of ethidium bromide solution (1µg/ml) was added to each sample.

The samples were then subjected to electrophoresis on 1% (w/v) agarose gels containing 0.65 M formaldehyde in MOPS buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate, 2 mM EDTA, pH 8.0). Gels were run for 3-4 h at 80 V with MOPS buffer as running buffer. To prepare for the transfer, gels were first soaked in 10 x SSC twice for 20 min. The RNA was then blotted onto nitrocellulose membrane (BA83, Schleicher & Schüll) by overnight capillary transfer, using 20 x SSC as transfer buffer. Afterwards the membrane was washed in 6 x SSC, sandwiched between 3MM paper (Whatman), and baked in a vacuum oven at 80°C for 2 h to fix the RNA to the nitrocellulose. The membrane was prehybridized in 10 ml of RNA hybridization solution (0.5 M $NaHPO_4$, pH 7.2, 1 mM EDTA, 1% [w/v] BSA, 7% [w/v] SDS) for 2 h at 60°C in a hybridization oven (Hybaid). Hybridization was carried out at 60°C overnight in 10 ml of the same solution containing the DNA probe. Probes (listed in Table 3) were generated by PCR and subsequently labeled with [α-^{32}P] dATP (Amersham) using the Random Primed DNA Labeling Kit (Boehringer Mannheim).

Table 3: *Probes used in Northern blots*

Probe	Size of ORF (kb)	Size of probe (kb)[1]
ADH2	1.04	1.04
HSP12	0.33	0.33
HSP26	0.64	0.64
SSA3	1.95	1.95
SSB1	1.84	1.84
RIM15-S[2]	5.3	0.52
RIM15-L[2]	5.3	1.30
TPS2	2.69	2.69
UBI4	1.14	1.14

[1] The sequences of the primers used to generate the probes are listed in Table 2.
[2] *RIM15*-S refers to the short (0.52 kb) probe, *RIM15*-L to the long (1.30 kb) probe used for Northern blots (see text).

Flow cytometry

For the determination of cell cycle phase during growth, cells were analyzed by flow cytometry. Samples were prepared following the protocol of Helliwell *et al.* (1994). In detail, 300 µl of cells (1-2*10^7 cells/ml, denser cultures were diluted) were sonicated for 2 min in a waterbath, and fixed by addition of 700 µl ethanol. Samples were incubated over night (or until all samples had been collected) at 4°C. Cells were then pelleted, washed with TE and resuspended in 500 µl Na-citrate (50 mM) containing RNase (0.25 mg/ml). To avoid aggregation of cells, the suspension was again sonicated for 2 min in a waterbath and then incubated for 1 h at 37°C to digest the RNA. DNA was stained by adding 500 µl of Na-citrate (50 mM) containing 16 µg/ml propidium iodide (Fluka). Samples were kept in the dark until analysis. For each sample 10'000 events were analyzed for DNA content with a Becton Dickinson FACScan (Lincoln Park, NJ).

The propidium iodide stained samples were also used to determine the cell cycle phase of budded cells. For this purpose, cells were examined under the microscope and budded cells were categorized as being (i) *before* nuclear division (1 nucleus in mother cell, no nucleus in daughter cell), (ii) *in* nuclear division (dividing nucleus in mother cell), or (iii) *after* nuclear division (both mother and daughter cell contain a nucleus).

Heat shock and other stress conditions

Cells growing exponentially (5*10^6 - 1*10^7 cells/ml) at 27°C in liquid medium were subjected to a heat shock at 42°C in a shaking water bath. For the determination of enzyme activities and trehalose content samples were harvested after 1 h. The time zero sample was taken before the transfer to 42°C. Other stress treatments were performed as follows: (i) osmotic shock: 5 M NaCl stock solution was added to the culture to yield a final concentration of 0.3 M, (ii) methanol stress: methanol was added to the cultures to a final concentration of 10% (v/v), and (iii) oxidative stress: H_2O_2 was added to the cultures to a final concentration of 0.3 mM H_2O_2. Samples were taken at the times indicated in the figure legend. The time zero samples were taken immediately after the addition of the different agents.

Determination of thermotolerance

For the determination of thermotolerance, aliquots of the cultures (1 ml) were transferred to prewarmed glass tubes, incubated at 50°C-53°C for 4-20 min (see table legends for exact conditions of this 'challenging heat shock' for each strain), rapidly cooled on ice, appropriately diluted with sterile water and plated on YPD agar. Colonies were

counted after 3 d at 27°C and the percentage of survival was assessed by comparison with controls not subjected to the challenging heat shock (100%).

Enzyme assays

Tre6P synthase, Tre6P phosphatase, and neutral trehalase

Activities of Tre6P synthase, Tre6P phosphatase and neutral trehalase were assayed in permeabilized cells as described before (De Virgilio *et al.*, 1991a). Log-phase (10 ml) or stationary phase (1 ml) cells were harvested by filtration on GF/C filters (Whatman), washed quickly with ice cold water and resuspended in 1 ml of lysis buffer (0.2 M Tricine, pH 7.0, 0.05% [v/v] Triton X-100 for Tre6P synthase and neutral trehalase; 50 mM KH_2PO_4, pH 6.0, 0.05% [v/v] Triton X-100 for Tre6P phosphatase). The suspensions were then frozen rapidly in liquid nitrogen and either analyzed immediately or stored at -20°C. Before performing the assays cells were thawed at 30°C for 2-3 min and then washed twice with lysis buffer lacking Triton X-100. Tre6P synthase was measured at 50°C using the coupled assay as described by Hottiger *et al.* (1987). Activity of the Tre6P phosphatase was determined at 30°C by incubation of permeabilized cells with Tre6P (Sigma) and quantification of the product trehalose by high performance liquid chromatography (HPLC) as described below. Trehalase activity was measured at 30°C by incubation of cells with trehalose and quantification of glucose released by using the GOD/POD kit (Boehringer Mannheim) (De Virgilio *et al.*, 1991a).

Invertase

Invertase activity was assayed in cell free extracts by adding sucrose and determining the glucose released (Goldstein and Lampen, 1975). Extracts were prepared from 100 ml of log-phase or 10 ml of stationary phase cells. The washed cells were resuspended in 500 µl of Tricine buffer (0.2 M, pH 7.0) and 1.5 g of glass beads (Ø 0.5 mm) were added. Cells were broken by vortexing three times for 1 min, interrupted by 1 min cooling on ice between vortexing. The resulting crude extracts were then centrifuged for 10 min at 25'000 *g* at 4°C and invertase activity assayed in the supernatants. 20 µl of appropriately diluted supernatant were mixed with 40 µl of sucrose (200 mM) and 340 µl of Na-acetate (50 mM, pH 5.0). Controls were prepared without addition of sucrose. The samples were incubated at 37°C for 10-30 min, boiled to stop the reaction and centrifuged. The glucose concentration in the supernatant was quantitated by means of the GOD/POD kit of Boehringer Mannheim.

β-Galactosidase

The activity of β-galactosidase was determined in permeabilized cells using *o*-nitrophenyl-β-D-galactoside (ONPG) as substrate (Miller, 1972). About 600 µl of culture were pelleted, washed once with Z-buffer (40 mM NaH_2PO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) and resuspended in 1 ml of Z-buffer. 100 µl of the cell suspension was used for the assay, from the remaining suspension the OD600 was determined. For the assay, 700 µl of Z-buffer containing 0.27% (v/v) β-mercapto-ethanol (freshly prepared each time), 40 µl chloroform and 20 µl SDS (0.1% [w/v]) were added to the cell suspension. The suspension was vortexed well and then incubated with 200 µl of ONPG (4 mg/ml) at 30°C for 5-30 min. The reaction was stopped with 400 µl of Na_2CO_3 (1M). After spinning the samples for 3 min the OD420 was measured and the β-galactosidase activity calculated as Miller Units: OD420*(OD600*volume of cell suspension in assay [l]*incubation time [min])$^{-1}$= β-galactosidase activity.

Quantification of trehalose

For the determination of trehalose, 10 ml of log-phase or 1-2 ml of stationary phase cells were filtered using GF/C filters (Whatman), washed twice with 5 ml of H_2O, resuspended in 1 ml of H_2O and boiled for 10 min. Extracts were cleared by three times centrifuging for 10 min at 20'000 *g*. The trehalose concentration was measured in the supernatant by HPLC analysis as described (De Virgilio *et al.*, 1993), using an anion-exchange column (CarboPac PA-1, Dionex) and a Dionex DX-300 Gradient Chromatography System. Peaks were detected with a pulsed amperometric detector (Dionex).

Quantification of glycogen

Glycogen contents were determined according to Lillie and Pringle (1980) by hydrolyzing the glycogen with α-amyloglucosidase (Fluka) and determining the amount of the resulting glucose. 10-20 ml of cells from upper log or stationary phase were pelleted, washed twice with H_2O and resuspended in 1 ml Na_2CO_3 (0.25 M). The samples were boiled for 1 h in tightly closed tubes and then cooled on ice. 200 µl of the suspension were mixed with 50 µl of acetic acid (3 M) and 700 µl of sodium acetate buffer (0.2 M, pH 4.8) and 50 µl of α-amyloglucosidase (10 U) were added. The suspension was incubated at 37°C for 22 h and the reaction stopped by boiling the samples for 5 min. After spinning the samples, glucose was determined in the supernatant with the GOD/POD kit (Boehringer Mannheim).

Quantification of protein

Protein concentrations in cell free extracts were measured using the Bio-Rad protein assay according to the manufacturer's instructions, using BSA as the standard. Total protein concentrations in the cultures were determined by means of a modified Lowry assay (Peterson, 1977) using BSA as the standard.

Purification of epitope-tagged proteins from *E. coli*

GST-Tps1 and GST

From overnight cultures of *E. coli* containing plasmid pGEX3X-*TPS1* or pGEX3X 15 ml were inoculated into 500 ml LB medium with ampicillin (75 mg/l) and grown until an OD600 of 0.5. Expression of GST-Tps1 or GST was induced by adding IPTG to a final concentration of 0.3 mM and subsequent growth for 3 h. The cultures were harvested by centrifugation for 5 min at 4'000 x *g* at 4°C and washed once with 100 ml STE (25% [w/v] sucrose, 25 mM Tris-HCl, pH 8.0, 1 mM EDTA). The pellets were then resuspended in 30 ml STE. After addition of 3 ml lysozyme (10 mg/ml) cells were incubated on ice for 30 min. The cell suspensions were mixed with 12 ml 1% (v/v) Nonidet P-40 containing protease inhibitors (1 tablet of Complete™ protease inhibitor cocktail per 50 ml) and then sonicated for 30 s, incubated on ice for 1 min and once more sonicated for 10 s to break the cells. The lysates were centrifuged for 10 min at 16'000 x *g* at 4°C. In the meantime the glutathione sepharose columns (2 ml bed volume, Pharmacia Biotech) were washed with 20 ml PBS (135 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and then equilibrated with 10 ml PBS with 1% Triton X-100. 25 ml of the soluble fraction of the lysates were combined with 92.75 ml H_2O, 6.25 ml 20x PBS, and 1 ml Triton X-100 and then loaded onto the columns at a flow rate of about 1 ml/min. The fusion proteins bind to the resin because of the strong affinity of glutathione S-transferase to glutathione. The columns were washed with 20 ml PBS to remove nonspecifically bound proteins. Eventually the bound GST-fusion proteins were eluted from the columns with 20 ml Tris-HCl (50 mM, pH 8.0) containing 5 mM glutathione and twenty 1-ml fractions were collected. 14 fractions containing the protein peak were pooled and the eluates concentrated 25 times by centrifugation in Centriprep 30 tubes (exclusion limit 30 kDa; Amicon) according to the instructions provided by the manufacturer. The concentrated eluates were then mixed with an equal volume of glycerol and stored at -20°C. GST alone was not concentrated because of its size (26 kDa) being lower than the exclusion limit of the Centriprep tubes.

MBP-Tps1, MBP-Tpk1

E. coli cells transformed with either pMAL-*TPS1* or pMAL-*TPK1* were grown, induced by IPTG and lysed as described for GST-fusion proteins above. The crude extracts were diluted 5 times with column buffer (10 mM sodium phosphate, 0.5 mM NaCl, 1 mM sodium azide, 10 mM 2-mercaptoethanol, 1 mM EGTA) containing 0.25% Tween 20. Amylose resin columns (2 ml bed volume, New England Biolabs) were washed with 10 ml column buffer and then equilibrated with 20 ml column buffer/0.25% Tween 20. The diluted crude extracts were loaded onto the columns at a flow rate of 1 ml/min. MBP-fusion proteins bind to the resin because of MBP's affinity for amylose. Nonspecifically bound proteins were washed off the columns by rinsing with 20 ml column buffer/0.25% Tween 20 and then with 10 ml column buffer alone. Subsequently, the MBP-fusion proteins were eluted from the resin with 20 ml column buffer containing 10 mM maltose and twenty 1-ml fractions were collected. The 14 fractions containing the protein peak were then pooled and the proteins concentrated as described for GST-fusion proteins. MBP alone as a control was either commercially obtained (New England Biolabs) or received as a gift from Carla Köhler (Biocenter, Basel).

Coprecipitation experiments

Purification of GST-Rim15

Strain MH272-1D transformed with either YCpIF2-*GST-RIM15* or YCpIF2-*GST* (control) was grown for 4 d on 500 ml SD medium with 2% (w/v) galactose and 1% (w/v) raffinose to induce *GAL1*-driven expression of *GST-RIM15* and *GST*, respectively. Cells were then harvested by centrifugation at 4°C, washed once with water, once with lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% [v/v] Nonidet P-40, and 1 tablet of Complete™ protease inhibitor cocktail per 50 ml [Boehringer Mannheim]) and then resuspended in 5 ml lysis buffer in 50 ml screw cap tubes. Glass beads (Ø 0.5 mm) were added and cells broken by 5 cycles of vigorous shaking by hand and cooling on ice for 1 min each. The extracts were precleared by centrifugation at 4'000 x *g* for 5 min at 4°C and the supernatants clarified by three cycles of centrifugation at 25'000 x *g* for 10 min at 4°C. 50 µl/ml glutathione sepharose 4B (Pharmacia Biotech) was added to each extract (50-70 mg total protein) and left to bind for 3-4 h at 4°C with gentle agitation. Afterwards the glutathione sepharose with the bound GST-Rim15 or GST was pelleted and the resin washed four times with lysis buffer and then three times with 50 mM Tris, pH 8.0, removing the supernatants completely with a Hamilton pipette. Eventually pellets were resuspended in 1 ml 50 mM Tris, pH 8.0 and the suspensions divided: 500 µl were used for the pull-down experiments

with glutathione sepharose, 500 µl for the pull-down experiments with amylose resin (New England Biolabs).

Pull-down with glutathione sepharose

An aliquot (50 µl) of the suspension was boiled with 5x SDS-gel loading buffer and loaded on an SDS-gel to check for recovery of GST or GST-Rim15. The remaining suspension was equally distributed between four reaction tubes. To the first and second tube 5 µg of a putative interactor (MBP-Tps1 or MBP-Tpk1, respectively), to the third and fourth tube 5 µg of an unrelated control (MBP or MBP-Tim12, respectively; gifts of Carla Köhler, Biocenter, Basel) were added together with 1 ml lysis buffer. The samples were incubated over night at 4°C with gentle agitation in order to allow binding of the proteins to each other. The sepharose was pelleted and washed as described above and each pellet was boiled in 30 µl of 5x SDS-gel loading buffer. From each sample an aliquot of 10 µl was loaded onto two separate SDS-gels (10%; Laemmli, 1970). Immunoblot analysis was performed as described below.

Pull-down with amylose resin

For the amylose resin pull-down the proteins (GST-Rim15, GST) first had to be eluted from the glutathione sepharose. This was done by adding 150 µl of 50 mM Tris-HCl, pH 8.0 with 5 mM glutathione to the sepharose pellets, mixing, and incubation on ice for 15 min. The sepharose was then pelleted and the supernatants containing GST-Rim15 or GST removed. An aliquot (10 µl) of the supernatants was mixed with SDS-gel loading buffer and loaded on an SDS-gel to check for recovery of the proteins. The remaining eluates of either GST or GST-Rim15 were distributed between four reaction tubes each and 5 µg of the different MBP-fusion proteins were added as described above. 20 µl of amylose resin were pipetted into each tube and the samples incubated over night at 4°C with gentle agitation. Further handling of the samples has been outlined above, immunoblot analysis was performed as described below.

Immunoblot analysis

After SDS-PAGE, gels were blotted onto nitrocellulose membrane (BA83, Schleicher & Schüll) in an electro-blotting unit with transblot buffer (25 mM Tris, 125 mM glycine, and 15% methanol) for 1 h 15 min at 40 V and 4°C. In the case of the amylose resin pulldown experiment, blotting time was extended to 1 h 45 min in order to ensure that potentially interacting GST-Rim15 protein would be transferred completely to the membrane. After the blotting, nitrocellulose membranes were blocked for 1 h at room temperature in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 3% (w/v)

BSA. Subsequently, membranes were incubated for at least 1 h in TTBS (TBS with 0.05% [v/v] Tween 20) containing 1% (w/v) BSA and either anti-GST (dilution 1:1000; Upstate Biotechnology Inc.) or anti-MBP (dilution 1:5000; New England Biolabs) antibodies. If membranes were incubated overnight, this was done at 4°C. The membranes were then washed twice with TTBS. The second antibodies (goat anti-rabbit-alkaline phosphatase conjugate, dilution 1:10'000; Sigma) were added in TTBS with 1% (w/v) BSA and membranes were incubated for 1-2 h at room temperature. Subsequently membranes were washed twice with TTBS and once with TBS. To develop the bands, alkaline phosphatase color development buffer (100 mM Tris-HCl, pH 9.5, 1 mM MgCl), 45 µl NBT (75 mg/1 ml 70% [v/v] DMF), and 35 µl X-phosphate (50 mg/1 ml DMF) were mixed and added to the membranes in the dark. Reactions were stopped after 30 min, or when bands were sufficiently visible, by rinsing the membranes thoroughly with water.

Kinase assays

For kinase assays the various GST-Rim15-fusions and GST (control) were purified essentially as described above for the coprecipitation assays, except that protein-bound sepharose pellets were washed four times with lysis buffer and subsequently three times with kinase buffer (50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 1 tablet of Complete™ protease inhibitor cocktail per 50 ml). Aliquots of the pellets (typically 10-15 µl of sepharose) were then incubated in the presence of kinase buffer containing phosphatase inhibitors (50 mM NaF, 10 mM Na-orthovanadate, 15 mM p-NO_2-phenylphosphate, 50 mM β-glycerophosphate, and 5 mM Na-pyrophosphate), and 10 µCi γ-[^{32}P] ATP, with or without α-casein (5 µg) as substrate in a total volume of 20 µl for 30 min at 30°C. By adding SDS-gel loading buffer and boiling for 5 min reactions were terminated. Samples were subjected to SDS-PAGE, gels were dried and eventually exposed on X-ray film or on a phosphoimager. Quantitation of ^{32}P was achieved using the Phosphor Analyst software (Bio-Rad).

When Tps1 was used as substrate for kinase assays with Rim15, the procedure was slightly altered. Assays were initially performed as described, except that 5 µg of MBP-Tps1 were added as substrate instead of α-casein. After incubation at 30°C samples were spun briefly in a microfuge to pellet the sepharose together with the bound GST-fusions. This was necessary because of the larger size of MBP-Tps1 compared to casein, making it difficult to decide whether bands on the autoradiograms came from phosphorylation of degradation products of Rim15 or from phosphorylation of Tps1. Supernatants, containing the MBP-Tps1 were transferred to fresh tubes. Both, supernatants and pellets were boiled with SDS-gel loading buffer and subjected to SDS-PAGE. Gels were dried and exposed as described.

To assay *in vitro* autophosphorylation and phosphorylation of Rim15 by bovine cAPK equal amounts of protein-bound glutathione sepharose pellets (10-15 µl) were incubated for 30 min at 30°C in the presence or absence of 1 U A kinase catalytic subunit (Sigma) and/or A kinase Inhibitor (Sigma) in a total volume of 20 µl of kinase buffer containing phosphatase inhibitors and 10 µCi γ-[^{32}P] ATP. Assays were stopped and samples analyzed by SDS-PAGE and autoradiography as described above. If the influence of cAPK-dependent phoshorylation of Rim15 on its kinase activity was to be assessed, reactions were incubated in the presence or absence of 1 U A kinase catalytic subunit as above but without γ-[^{32}P] ATP. Reactions were then terminated by washing the sepharose pellets three times with kinase buffer containing phosphatase inhibitors. The pellets were then incubated with α-casein and γ-[^{32}P] ATP and subjected to SDS-PAGE and autoradiography analysis as described above.

The possibility of phosphorylation of Tps1 by bovine cAPK *in vitro* was tested by incubating 5 µg of GST-Tps1 purified from bacteria with 1 U of A kinase catalytic subunit, with or without A kinase Inhibitor, in kinase buffer containing phosphatase inhibitors, and 10 µCi γ-[^{32}P] ATP in a total volume of 20 µl. Samples were incubated for 30 min at 30°C and reactions terminated and analyzed by SDS-PAGE and autoradiography as described above.

CHAPTER I

STRUCTURAL ANALYSIS OF THE SUBUNITS OF THE TREHALOSE-6-PHOSPHATE SYNTHASE/PHOSPHATASE COMPLEX IN *SACCHAROMYCES CEREVISIAE* AND THEIR FUNCTION DURING HEAT SHOCK

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Structural analysis of the subunits of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae* and their function during heat shock

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Summary

Synthesis of trehalose in the yeast *Saccharomyces cerevisiae* is catalysed by the trehalose-6-phosphate (Tre6*P*) synthase/phosphatase complex, which is composed of at least three different subunits encoded by the genes *TPS1*, *TPS2*, and *TSL1*. Previous studies indicated that Tps1 and Tps2 carry the catalytic activities of trehalose synthesis, namely Tre6*P* synthase (Tps1) and Tre6*P* phosphatase (Tps2), while Tsl1 was suggested to have regulatory functions. In this study two different approaches have been used to clarify the molecular composition of the trehalose synthase complex as well as the functional role of its potential subunits. Two-hybrid analyses of the *in vivo* interactions of Tps1, Tps2, Tsl1, and Tps3, a protein with high homology to Tsl1, revealed that both Tsl1 and Tps3 can interact with Tps1 and Tps2; the latter two proteins also interact with each other. In addition, trehalose metabolism upon heat shock was analysed in a set of 16 isogenic yeast strains carrying deletions of *TPS1*, *TPS2*, *TSL1*, and *TPS3* in all possible combinations. These results not only confirm the previously suggested roles for Tps1 and Tps2, but also provide, for the first time, evidence that Tsl1 and Tps3 may share a common function with respect to regulation and/or structural stabilization of the Tre6*P* synthase/phosphatase complex in exponentially growing, heat-shocked cells.

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Introduction

The non-reducing disaccharide trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is widespread in nature and has been found in many prokaryotes, fungi, and some lower plants and animals (Elbein, 1974). Interestingly, trehalose synthesis is induced in most of these organisms in response to a small set of specific environmental conditions. In particular, trehalose is accumulated in phases of nutrient starvation, desiccation, and after exposure to a mild heat shock (for reviews see Van Laere, 1989; Wiemken, 1990; Crowe *et al.*, 1992). It has been suggested, therefore, that trehalose plays a role as a stabiliser of cellular structures under stress conditions (Keller *et al.*, 1982; Crowe *et al.*, 1984). In accordance with this suggestion, *in vitro* studies have revealed the exceptional capability of trehalose in protecting biological membranes and enzymes from freezing- or drying-induced dehydration (for a review see Crowe *et al.*, 1992) and heat stress (Hottiger *et al.*, 1994).

As early as 1958 (Cabib and Leloir, 1958) trehalose biosynthesis was found to be catalysed by a two-step process involving trehalose-6-phosphate (Tre6*P*) synthase and Tre6*P* phosphatase. Since then, several studies have dealt with the purification and kinetic properties of Tre6*P* synthase and Tre6*P* phosphatase in yeast (Panek *et al.*, 1987; Vandercammen *et al.*, 1989; Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Londesborough and Vuorio, 1993). The overall picture that emerged from these studies is that both enzymes are part of a multimeric protein complex with an approximate molecular mass of 630–800 kDa. This complex, the Tre6*P* synthase/phosphatase complex, is composed of at least three different subunits with apparent molecular masses of 56, 102, and 123 kDa. The corresponding genes, *TPS1* (encoding the 56 kDa subunit; Bell *et al.*, 1992), *TPS2* (encoding the 102 kDa subunit; De Virgilio *et al.*, 1993), and *TSL1* (encoding the 123 kDa subunit; Vuorio *et al.*, 1993), have been identified and sequenced. Based on its homology to Tsl1 (55% identity upon optimal alignment over the entire amino acid sequence; Manning *et al.*, 1992), Tps3 may constitute a fourth subunit of the complex. Remarkably, the deduced amino acid sequence of *TPS1* shows striking similarity over its

entire sequence to parts of the deduced amino acid sequences of all three (*TPS2*, *TSL1*, and *TPS3*).

Several observations indicate that Tps1 may carry the catalytic activity of the Tre6*P* synthase: (i) *tps1*Δ strains lack any detectable Tre6*P* synthase activity (Bell *et al.*, 1992); (ii) *TPS1* expression in *Escherichia coli* (Vuorio *et al.*, 1993) and in yeast (W. Bell *et al.*, submitted) results in greatly increased Tre6*P* synthase activity; (iii) *TPS1* complements the trehalose-synthesis defect of an *E. coli otsA* mutant (McDougall *et al.*, 1993); and (iv) transgenic tobacco plants expressing *TPS1* are able to synthesize trehalose (Holmström *et al.*, 1996). It was therefore surprising that *TPS1* was found to be identical to the previously sequenced *CIF1* (catabolite inactivation of fructose-1,6-bisphosphatase), a gene reported to be essential for growth on glucose in *Saccharomyces cerevisiae* (González *et al.*, 1992). Several mutations described earlier as *fdp1*, *cif1*, *byp1*, and *glc6* have been shown to be alleles of the same gene and to exert a wide range of pleiotropic phenotypes (reviewed in Thevelein and Hohmann, 1995). An important phenotype of all these mutants (except *glc6*), however, is that they are unable to grow on glucose, apparently because of an uncontrolled influx of glucose into the glycolytic pathway (Van Aelst *et al.*, 1991; Hohmann *et al.*, 1993; Blázquez *et al.*, 1993; Blázquez and Gancedo, 1994). At least three different models for this unexpected role of Tps1 (also called Ggs1 in this context) in the control of glycolysis have been presented, as follows. (i) Trehalose synthesis could serve as a metabolic buffer system by draining off excessively synthesized sugar phosphates into trehalose and thereby releasing phosphate which is required further along the glycolytic pathway for the glyceraldehyde-3-phosphate dehydrogenase reaction. (ii) Tre6*P* has been shown to inhibit *S. cerevisiae* hexokinases *in vitro* and its absence in *tps1*Δ strains could result in unrestrained activity of hexokinases, leading to an excessive flux of glucose through the early steps of glycolysis. (iii) Finally, Tps1 could directly regulate sugar transport and/or sugar phosphorylation, in addition to its role as a subunit of the Tre6*P* synthase/phosphatase complex (see Thevelein and Hohmann, 1995, for a review of these models).

Disruption of *TPS2* causes loss of Tre6*P* phosphatase activity and accumulation of Tre6*P* under conditions in which wild-type cells normally accumulate trehalose (heat shock and stationary phase), suggesting that Tps2 carries the catalytic activity of Tre6*P* phosphatase (De Virgilio *et al.*, 1993). While *TPS1* and *TPS2* encode Tre6*P* synthase and Tre6*P* phosphatase, respectively, the role of the *TSL1* (for Tre6*P* synthase long chain) gene product with respect to trehalose synthesis is not yet understood. It was reported that partial proteolytic degradation of Tsl1, the 123 kDa subunit of the complex, reduces the phosphate-mediated inhibition as well as the fructose-6-phosphate-mediated activation of Tre6*P* synthase (Vuorio *et al.*, 1993). Thus, Tsl1 may be involved in modification of the kinetic properties of Tre6*P* synthase activity. Recent studies on *TPS3* and *TSL1* expression levels suggest that Tsl1 and Tps3 may to some extent act as interchangeable regulators of the Tre6*P* synthase/phosphatase complex, but that they also may exert different regulation under specific growth conditions (Winderickx *et al.*, 1996). The precise function of both proteins, however, is not clear. The present study was undertaken in order to clarify the molecular composition of the Tre6*P* synthase complex as well as the functional role of its potential subunits. Our results using the two-hybrid system (Fields and Sternglanz, 1994) show that both Tsl1 and Tps3 can interact with Tps1 and Tps2 *in vivo*, while the latter two proteins also interact with each other. In addition, we show, by analysis of the heat-induced responses of trehalose metabolism in deletion mutants, that all four potential subunits indeed have a functional role in the synthesis of trehalose.

Results

Two-hybrid analysis of interactions between Tps1, Tps2, Tps3, and Tsl1

A variety of biochemical data suggest that Tps1, Tps2, and Tsl1 interact intimately with each other to form the Tre6*P* synthase/phosphatase complex (Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Vuorio *et al.*, 1993; Londesborough and Vuorio, 1993). Based on its homology to Tsl1, a fourth protein, Tps3, may also be part of this complex (De Virgilio *et al.*, 1993). To investigate the possible interactions of these four proteins, directed two-hybrid analysis (Fields and Sternglanz, 1994) was performed. Strong interactions (based on β-galactosidase activities) were determined for all possible combinations of pairs between Tps1-AD, Tps2-AD, and Tps3-AD and Tps1-DBD, Tps2-DBD, and Tps3-DBD (Table 1). Thus, Tps1, Tps2, and Tps3 appear to interact not only directly with each other, but also with themselves, suggesting that each of these subunits may be present as a homodimer or homomultimer in the Tre6*P* synthase/phosphatase complex. It must be pointed out that these data provide the first experimental evidence that Tps3 may in fact be a part of this complex.

As a full-length Tsl1-AD did not interact with any of the other Tps-DBD fusions (data not shown), we tested a truncated Tsl1-AD (Tsl1-I-AD; amino acids 60–332), which was originally isolated in a two-hybrid screen for proteins interacting with Tps2, for interaction with the other proteins. Accordingly, Tsl1-I-AD showed weak interaction with Tps1-DBD, strong interaction with Tps2-DBD, and very weak or no interaction with Tps3-DBD and Tsl1-DBD. The full-length Tsl1-DBD interacted weakly with both Tps1-AD and Tps2-AD, but not with Tps3-AD or

Table 1. Two-hybrid interactions among the subunits of the trehalose-6-phosphate synthase complex in *Saccharomyces cerevisiae*.

	DBD Fusions					
AD fusions	*TPS1*	*TPS2*	*TPS3*	*TSL1*	*MSB2*	pEG202
TPS1	**548**	**150**	**696**	**49**	1	5
TPS2	**208**	**528**	**411**	16	2	17
TPS3	**708**	**239**	**425**	3	18	8
TSL1-I	**53**	**346**	20	3	12	6
pJG4–5	2	1	3	2	18	10

Possible interactions among Tps1, Tps2, Tps3, and Tsl1 were detected using the two-hybrid system described in the *Experimental procedures*. Numbers represent mean β-galactosidase activities (in Miller units) from three independent transformants for each pair of plasmids. *TSL1-I* codes for an internal part of Tsl1 (amino acids 60–332). pJG4-5 indicates the AD vector with no insert; pEG202 is the DBD vector with no insert. Values that were at least 20-fold higher than the corresponding control (pJG4-5 without insert) are shown in bold.

Tsl1-I-AD. Thus, Tps3 and Tsl1 appear to interact with both Tps1 and Tps2, but not with each other.

Effects of TPS1, TPS2, TPS3, *and* TSL1 *deletions*

To determine the consequences of the loss of Tps1, Tps2, Tps3, and Tsl1 for trehalose metabolism during heat shock, we replaced the complete *TPS1*, *TPS2*, *TPS3*, and *TSL1* coding sequences in the wild-type strain YSH 6.106.-3A with *TRP1*, *LEU2*, *URA3*, and *HIS3*, respectively. An isogenic set of 15 strains carrying these four deletions in all possible combinations was constructed as described in the *Experimental procedures*. While strains with an intact Tps1 were able to grow on glucose, all *tps1*Δ strains were defective for growth on glucose, irrespective of additional mutations in *TPS2*, *TPS3*, or *TSL1*, or any combination of these deletions (W. Bell *et al.*, submitted). As none of the strains was defective for growth on galactose, the following experiments were all carried out on media containing galactose as carbon source. The 15 strains and their isogenic wild-type strain YSH 6.106.-3A were grown to exponential phase at 27°C, subjected to a heat shock for 1 h at 42°C, and assayed for their trehalose and Tre6*P* contents as well as for their activities of Tre6*P* synthase and Tre6*P* phosphatase (Table 2). As expected, wild-type cells had very high Tre6*P* synthase (0.543 μkat g^{-1} protein) and Tre6*P* phosphatase (0.051 μkat g^{-1} protein) activities *in vitro* and contained high levels of trehalose (0.327 g g^{-1} protein) but very low levels of Tre6*P* (<0.010 g g^{-1} protein) *in vivo* (Table 2, row 1).

Previous studies suggested that the catalytic subunits of Tre6*P* synthase and Tre6*P* phosphatase are encoded by *TPS1* and *TPS2*, respectively (Bell *et al.*, 1992; De Virgilio *et al.*, 1993). In accordance with these suggestions, all strains harbouring *tps1*Δ were found to have virtually no Tre6*P* synthase activity and to be unable to accumulate detectable amounts of trehalose or Tre6*P* during heat shock (Table 2, rows 2, 6, 7, 8, 12, 13, 14, and 16). In addition, all strains carrying *tps2*Δ had drastically reduced Tre6*P* phosphatase activities and were found to accumulate high levels of Tre6*P* during heat shock (between 0.257 and 0.199 g g^{-1} protein) provided that they contained a functional *TPS1* (Table 2, rows 3, 9, 10, and 15). A residual level of Tre6*P* phosphatase activity (below 9 nkat g^{-1} protein) was found in all *tps2*Δ strains including

Table 2. Trehalose and trehalose-6-phosphate levels and activities of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in heat-shocked wild-type yeast and mutants with various combinations of *tps1*Δ, *tps2*Δ, *tps3*Δ, and *tsl1*Δ.

Row no.	Strain	Relevant genotype	Trehalose (g g^{-1} protein)	Tre6*P* (g g^{-1} protein)	Tre6*P* synthase (μkat g^{-1} protein)	Tre6*P* phosphatase (μkat g^{-1} protein)
1	YSH 6.106.-3A	Wild type	0.327 ± 0.016	< 0.010	0.543 ± 0.133	0.051 ± 0.002
2	YSH 6.106.-1A	*tps1*Δ	< 0.005	< 0.010	< 0.001	0.015 ± 0.006
3	YSH 6.106.-8C	*tps2*Δ	0.022 ± 0.003	0.199 ± 0.041	0.069 ± 0.036	0.004 ± 0.002
4	YSH 6.106.-2B	*tps3*Δ	0.344 ± 0.014	< 0.010	0.336 ± 0.083	0.051 ± 0.016
5	YSH 6.106.-19B	*tsl1*Δ	0.352 ± 0.056	< 0.010	0.427 ± 0.029	0.055 ± 0.014
6	YSH 6.106.-16D	*tps1*Δ *tps2*Δ	< 0.005	< 0.010	< 0.001	0.008 ± 0.007
7	YSH 6.106.-5B	*tps1*Δ *tps3*Δ	< 0.005	< 0.010	< 0.001	0.013 ± 0.005
8	YSH 6.106.-6A	*tps1*Δ *tsl1*Δ	< 0.005	< 0.010	< 0.001	0.014 ± 0.007
9	YSH 6.106.-19A	*tps2*Δ *tps3*Δ	0.020 ± 0.006	0.220 ± 0.020	0.031 ± 0.028	0.005 ± 0.001
10	YSH 6.106.-14B	*tps2*Δ *tsl1*Δ	0.015 ± 0.004	0.257 ± 0.010	0.027 ± 0.026	0.004 ± 0.001
11	YSH 6.106.-16C	*tps3*Δ *tsl1*Δ	0.128 ± 0.018	0.015 ± 0.010	0.011 ± 0.008	0.051 ± 0.006
12	YSH 6.106.-2D	*tps1*Δ *tps2*Δ *tps3*Δ	< 0.005	< 0.010	< 0.001	0.007 ± 0.004
13	YSH 6.106.-10A	*tps1*Δ *tps2*Δ *tsl1*Δ	< 0.005	< 0.010	< 0.001	0.008 ± 0.004
14	YSH 6.106.-8A	*tps1*Δ *tps3*Δ *tsl1*Δ	< 0.005	< 0.010	0.004 ± 0.003	0.008 ± 0.002
15	YSH 6.106.-1D	*tps2*Δ *tps3*Δ *tsl1*Δ	0.030 ± 0.013	0.252 ± 0.036	0.011 ± 0.005	0.008 ± 0.002
16	YSH 6.106.-4C	*tps1*Δ *tps2*Δ *tps3*Δ *tsl1*Δ	< 0.005	< 0.010	0.002 ± 0.002	0.005 ± 0.002

Log-phase cells of the individual strains were grown at 27°C, transferred to a water bath at 42°C, and incubated for 1 h. Results shown represent the mean ± SE of three to four independent experiments. Detection limits for the levels of trehalose, Tre6*P*, Tre6*P* synthase activity, and Tre6*P* phosphatase activity were 0.005 g g^{-1} protein, 0.010 g g^{-1} protein, 0.001 μkat g^{-1} protein, and 0.002 μkat g^{-1} protein, respectively.

the quadruple *tps1Δ tps2Δ tps3Δ tsl1Δ* strain (Table 2, rows 3, 6, 9, 10, 12, 13, 15 and 16), indicating that Tps1, Tps3, and Tsl1 are not responsible for this activity. The residual Tre6*P* phosphatase activity may be due to unspecific phosphatases.

Surprisingly, all strains carrying *tps1Δ* and a functional *TPS2* were not only defective for Tre6*P* synthase activity but also had greatly reduced levels of Tre6*P* phosphatase activity (Table 2, rows 2, 7, 8 and 14). Conversely, all strains carrying *tps2Δ* and a functional *TPS1* were not only defective for Tre6*P* phosphatase activity but also had significantly reduced levels of Tre6*P* synthase activity (Table 2, rows 3, 9, 10 and 15). Despite the low levels of *in vitro* detectable Tre6*P* synthase activity, all *TPS1 tps2Δ* strains were found to accumulate high amounts of Tre6*P* during heat shock (see above), indicating that their *in vivo* Tre6*P* synthase activities were sufficient to sustain this accumulation and are relatively unaffected by the loss of Tps2. Therefore, the low Tre6*P* synthase activities in *TPS1 tps2Δ* strains measured *in vitro* may be a reflection of an impaired structural integrity of the entire Tre6*P* synthase/phosphatase complex in extracts lacking Tps2. Similarly, the low activity of Tre6*P* phosphatase activity in *tps1Δ TPS2* strains may also be a result of the destabilization of the complex in extracts where Tps1 is absent. The triple deletion strain *tps2Δ tps3Δ tsl1Δ* (Table 2, row 15) was found to have low, but detectable, Tre6*P* synthase activity and to accumulate large amounts of Tre6*P*. Thus, the presence of Tps2, Tps3, and Tsl1 seems not to be a prerequisite for normal functioning of the Tre6*P* synthase *in vivo* under heat-shock conditions.

Earlier studies suggested that Tsl1 may be a regulatory protein which mediates fructose-6-phosphate activation and P_i inhibition of Tre6*P* synthase (Londesborough and Vuorio, 1993). Given the sequence similarity between Tsl1 and Tps3, both proteins may actually perform redundant regulatory functions or perform their functions under different physiological conditions. We found that deletion of *TPS3* (Table 2, row 4) resulted in only moderate reduction of Tre6*P* synthase (0.336 μkat g^{-1} protein) and did not affect the Tre6*P* phosphatase (0.051 μkat g^{-1} protein) activity when compared with the wild type. In accordance with these results, trehalose (0.344 g g^{-1} protein) and Tre6*P* (<0.010 g g^{-1} protein) levels also did not differ significantly from those in the wild-type strain (Table 2, row 1). Deletion of *TSL1* (Table 2, row 5) also resulted in a moderate reduction of Tre6*P* synthase activity (0.427 μkat g^{-1} protein) but no reduction of Tre6*P* phosphatase activity (0.055 μkat g^{-1} protein), and also had no significant effect on the amounts of trehalose (0.352 g g^{-1} protein) or Tre6*P* (<0.010 g g^{-1} protein) accumulated during heat shock, compared to the wild-type strain (Table 2, row 1). Thus, both single mutants, *tps3Δ* and *tsl1Δ*, were not significantly reduced in their Tre6*P* synthase activities or in their ability to accumulate trehalose. In contrast, deletion of both *TPS3* and *TSL1* (Table 2, row 11) was found to cause not only a dramatic reduction in Tre6*P* synthase activity (0.011 μkat g^{-1} protein) but also a significant reduction in the amount of accumulated trehalose (0.128 g g^{-1} protein) during heat shock, relative to the wild-type strain (Table 2, row 1). Tre6*P* phosphatase activity (0.051 μkat g^{-1} protein) and Tre6*P* levels (0.015 g g^{-1} protein) were not significantly altered in the *tps3Δ tsl1Δ* strain. These results indicate that Tps3 and Tsl1 indeed share a functional role with respect to the regulation of Tre6*P* synthase activity.

Discussion

A number of publications have dealt with the structural and functional analysis of the Tre6*P* synthase complex in *S. cerevisiae*. The biochemical and genetic data available suggest that the three proteins Tps1, Tps2, and Tsl1 form a multimeric protein complex (Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Vuorio *et al.*, 1993; Londesborough and Vuorio, 1993). In the present study we have performed a series of experiments to further elucidate the structural composition of the Tre6*P* synthase complex as well as the specific role of its potential subunits. Using the two-hybrid system, we show that these three proteins can physically interact with each other *in vivo*. A fourth protein with high homology to Tsl1, namely Tps3, was also found to interact with both Tps1 and Tps2 in the directed two-hybrid assay. Interestingly, while Tps1 and Tps2 interacted with all other subunits, including themselves, we could not detect any direct interaction between Tsl1 and Tps3, which may simply be explained by the potentially altered tertiary structure, lower protein stability, or low expression level of one of these two-hybrid constructs (see also Fields and Sternglanz, 1994). However, the absence of detectable Tsl1–Tps3 interaction may also indicate that binding of these two proteins to the Tre6*P* synthase complex may be either spatially or temporally separated, thus allowing the cells to regulate trehalose synthesis in response to a broader range of different physiological conditions. In accordance with such a model, *TPS3* and *TSL1* have been reported to be differentially expressed in *S. cerevisiae*. While *TPS3* is expressed at a constant rate in exponentially growing and stationary-phase cells, *TSL1* expression is greatly enhanced upon entrance into stationary phase (Winderickx *et al.*, 1996). Thus, the two proteins may indeed mediate, to some extent, differential regulation of the Tre6*P* synthase complex according to their relative abundance under different physiological conditions (e.g. exponential phase and stationary phase). Nevertheless, our finding that the *tps3Δ tsl1Δ* double mutant, in contrast to the corresponding single mutants, was seriously impaired in Tre6*P*

synthase activity indicates that both proteins must also share some common functions with respect to trehalose synthesis in exponentially growing, heat-shocked cells. In this context, it must be pointed out that our analysis of the *tps3Δ tsl1Δ* double mutant together with the results of the two-hybrid assays for the first time indicate that Tps3 is a subunit of the Tre6*P* synthase complex. Whether Tps3 is also involved in mediation of fructose-6-phosphate activation and P_i inhibition of the Tre6*P* synthase, as has been suggested for Tsl1 (Londesborough and Vuorio, 1993), and whether this regulation may vary according to the physiological conditions, remain to be elucidated.

Despite detailed biochemical analyses of the Tre6*P* synthase complex, the stochiometry of the various subunits is still unclear. Gel filtration experiments showed that the molecular mass of the complex is around 630–800 kDa (Londesborough and Vuorio, 1991; Bell *et al.*, 1992). As the sum of the molecular masses of the four putative subunits only adds up to ≈400 kDa, either some or all of the subunits must exist in more than one copy in the complex. In support of this hypothesis, we found that Tps1, Tps2, and Tps3 were able to interact with themselves in the two-hybrid system, indicating that they may be present as homodimers or homomultimers in the Tre6*P* synthase complex. Gel filtration experiments revealed that defined fractions representing proteins with an approximate molecular mass of 100 kDa contained only free Tps1 (56 kDa; W. Bell *et al.*, submitted), indicating that Tps1 may form a homodimer. Further detailed biochemical studies will be necessary to elucidate the exact stochiometry of the subunits of the Tre6*P* synthase complex and to determine whether the conclusions drawn from the results of our two-hybrid analyses reflect the situation *in vivo*. The interactions measured by the two-hybrid method now can be used to identify the domains on the subunits responsible for the interaction and, in particular, to find out whether the Tps1-homologous sections of Tps2, Tsl1, and Tps3 serve a function in the assembly of the complex.

Analysis of the isogenic set of strains carrying deletions of *TPS1*, *TPS2*, *TPS3*, and *TSL1* in all possible combinations confirmed the previously suggested roles for Tps1 and Tps2 (Bell *et al.*, 1992; De Virgilio *et al.*, 1993). As all strains deleted for *TPS1* lost their Tre6*P* synthase activity and were unable to accumulate trehalose or Tre6*P* during heat shock, we can conclude that *TPS1* codes for the Tre6*P* synthase. In addition, the finding that all *tps2Δ* strains had strongly reduced Tre6*P* phosphatase activities and accumulated high amounts of Tre6*P*, as long as they contained a functional *TPS1* gene, confirms that *TPS2* codes for the Tre6*P* phosphatase. Despite the absence of the specific Tre6*P* phosphatase activity, all *tps2Δ* strains including the quadruple *tps1Δ tps2Δ tps3Δ tsl1Δ* mutant were found to have residual Tre6*P* phosphatase activity. As none of the known subunits can be responsible for this residual activity in the quadruple deletion strain, we suggest that this activity is due to unspecific phosphatases. This would also explain the small amount of trehalose found in the *tps2Δ TPS1* strain.

A further interesting aspect of our studies is the finding that all strains deleted for *TPS1* have greatly reduced Tre6*P* phosphatase activities. Similar results were reported by Vuorio *et al.* (1993) and led to the speculation that Tps1 may also carry Tre6*P* phosphatase activity. This seems unlikely, however, as *tps2Δ* strains with an intact *TPS1* only accumulated residual amounts of trehalose (see above). It is therefore more likely that deletion of *TPS1* leads to a destabilization of the trehalose synthase complex, and hence to a decrease in Tre6*P* phosphatase activity, which is especially apparent under the conditions of the *in vitro* assay. Likewise, strains deleted for *TPS2* also lost most of their Tre6*P* synthase activity *in vitro*, even though they were able to synthesize Tre6*P* during heat shock, indicating that the Tre6*P* synthase was functional *in vivo*. Thus, it is possible that the absence of Tps2 also leads to destabilization of the Tre6*P* synthase complex, especially during the *in vitro* assay. In accordance with this suggestion, we previously found that Tre6*P* synthase activity in *tps2Δ* strains was not significantly reduced if determined in permeabilized cells (De Virgilio *et al.*, 1993), a method much less disruptive than the cell-extraction method used in this study. As particularly small proteins (<80 kDa, e.g. Tps1) may be lost during sample preparation in permeabilized cells (Miozzari *et al.*, 1978), use of cell extracts was clearly the method of choice in the present study, where the occurrence of free Tps1 in the extracts of some mutant strains could be anticipated. From these observations it is obvious that the Tre6*P* synthase activities measured *in vitro* often do not reflect the actual *in vivo* situation. A further example of the discrepancy between *in vitro* and *in vivo* activity of the Tre6*P* synthase is provided by the triple *tps2Δ tps3Δ tsl1Δ* mutant which has almost no detectable Tre6*P* synthase activity and yet is able to accumulate high amounts of Tre6*P* during a mild heat shock. This triple-deletion mutant is especially interesting as Tps1 seems to be able to synthesize a large amount of Tre6*P* even though it is probably present as a free subunit in this strain. Based on the finding that the *tps3Δ tsl1Δ* double mutant, in contrast to the *tps2Δ tps3Δ tsl1Δ* triple mutant, is seriously defective for Tre6*P* and trehalose synthesis one may speculate that the free Tps1 subunit may be negatively regulated by binding to Tps2. In this context, future studies should undoubtedly focus on the potential regulation of Tre6*P* synthase activity by the other subunits of the Tre6*P* synthase/phosphatase complex. In particular, it will be interesting to determine the exact biochemical

Table 3. List of strains used in this work.

Strain	Genotype	Source
YSH 6.106.-3A	α *leu2 ura3 trp1his3 ade2*	This work[a]
YSH 6.106.-1A	α *tps1Δ::TRP1*	This work[a]
YSH 6.106.-8C	α *tps2Δ::LEU2*	This work[a]
YSH 6.106.-2B	α *tps3Δ::URA3*	This work[a]
YSH 6.106.-19B	α *tsl1Δ::HIS3*	This work[a]
YSH 6.106.-16D	α *tps1Δ::TRP1 tps2Δ::LEU2*	This work[a]
YSH 6.106.-5B	α *tps1Δ::TRP1 tps3Δ::URA3*	This work[a]
YSH 6.106.-6A	α *tps1Δ::TRP1 tsl1Δ::HIS3*	This work[a]
YSH 6.106.-19A	α *tps2Δ::LEU2 tps3Δ::URA3*	This work[a]
YSH 6.106.-14B	α *tps2Δ::LEU2 tsl1Δ::HIS3*	This work[a]
YSH 6.106.-16C	α *tps3Δ::URA3 tsl1Δ::HIS3*	This work[a]
YSH 6.106.-2D	α *tps1Δ::TRP1 tps2Δ::LEU2 tps3Δ::URA3*	This work[a]
YSH 6.106.-10A	α *tps1Δ::TRP1 tps2Δ::LEU2 tsl1Δ::HIS3*	This work[a]
YSH 6.106.-8A	α *tps1Δ::TRP1 tps3Δ::URA3 tsl1Δ::HIS3*	This work[a]
YSH 6.106.-1D	α *tps2Δ::LEU2 tps3Δ::URA3 tsl1Δ::HIS3*	This work[a]
YSH 6.106.-4C	α *tps1Δ::TRP1 tps2Δ::LEU2 tps3Δ::URA3 tsl1Δ::HIS3*	This work[a]
EGY48	α *his3 trp1ura3 LEU2::pLexAop6-LEU2*	Zervos *et al.* (1993)

a. Isogenic to YSH 6.106.-3A except for the individual deletions. YSH 6.106.-3A is isogenic to, and a direct sibling of, W303-1A (a *leu-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*; Thomas and Rothstein, 1989).

nature as well as the physiological relevance of the suggested Tps3/Tsl1-mediated regulation of trehalose synthesis. Our two-hybrid studies as well as our functional analysis of the subunits of the trehalose synthase complex provide an excellent basis for such future studies.

Experimental procedures

Strains, media, and microbiological and recombinant DNA methods

The *S. cerevisiae* strains used in this study are listed in Table 3. *E. coli* strain JMB9 ((r^-m^+) Δ*trpF*) (Sterner *et al.*, 1995) was used to rescue pJG4-5-based plasmids from strain EGY48. The transformed cells were plated directly onto Vogel–Bonner minimal plates (Davis *et al.*, 1980) supplemented with 0.2% (w/v) glucose, 0.5% (w/v) casamino acid hydrolysate, 0.01 mM $FeCl_3$ and 100 mg ampicillin l^{-1}. Other plasmid manipulations were performed in *E. coli* strain DH5α (Gibco BRL) using standard procedures (Sambrook *et al.*, 1989). Standard procedures of yeast genetics and molecular biology (Guthrie and Fink, 1991; Sambrook *et al.*, 1989) were used. Yeast transformations were performed using a modification of the Li^+-ion method (Gietz *et al.*, 1992).

Yeast and *E. coli* media, including defined media (SD with appropriate amino acid supplements) were prepared according to standard recipes (Sambrook *et al.*, 1989; Rose *et al.*, 1990). Yeast cell cultures were incubated on a rotary shaker (140 r.p.m.) at 27°C, taking care that the cell densities were below 5×10^6 cells ml^{-1} at the beginning of the heat-shock experiments (1 h at 42°C).

The complete open reading frames of *TPS1*, *TPS2*, *TSL1*, and *TPS3* were deleted by the method of Eberhardt and Hohmann (1995). The strategies for deletion of *TPS1* (Van Aelst *et al.*, 1993) and *TPS2* (Hohmann *et al.*, 1996) have been described elsewhere. For deletion of *TSL1*, a plasmid carrying the *TSL1* open reading frame as well as 5′ and 3′ flanking sequences cloned into the *Sma*I site of pBluescript (Stratagene) (Vuorio *et al.*, 1993) was amplified by the polymerase chain reaction (PCR), using primers that yield a PCR product lacking the complete open reading frame. *Bgl*II sites that had been introduced in the primers were used to ligate this PCR product to a *Bam*HI–*HIS3* fragment, which was derived from the YDp set (Berben *et al.*, 1991). From the resulting plasmid a *Bam*HI–*Eco*RI fragment, containing *TSL1* flanking sequences separated by *HIS3* and *HIS3* flanking sequences, was isolated and used for yeast transformation. For the deletion of *TPS3*, a 4.2 kb *Xba*I–*Sma*I fragment containing the *TPS3* open reading frame including flanking sequences was cloned into the *Xba*I–*Sma*I sites of pUC19. The plasmid was amplified by PCR, using primers that yield a PCR product lacking the complete *TPS3* open reading frame. *Bam*HI sites that had been introduced into the primers were used to ligate this PCR product to a *Bam*HI–*URA3* fragment, which was derived from the YDp set (Berben *et al.*, 1991). From the resulting plasmid an *Xba*I–*Eco*RI fragment, containing *TPS3* flanking sequences separated by *URA3* and *URA3*-flanking sequences, was isolated and used for yeast transformation. All deletions were confirmed by Southern blot analysis. Strains carrying multiple deletions were generated by crossing and tetrad analysis.

Two-hybrid analyses

The interactions of the subunits of the Tre6*P* synthase complex were tested by two-hybrid analysis (Fields and Sternglanz, 1994), using the LexA system described in detail elsewhere (Gyuris *et al.*, 1993). To fuse the various full-length Tre6*P* synthase complex subunits to the LexA DNA-binding domain (DBD) coding sequences in plasmid pEG202 (Zervos *et al.*, 1993) and to the activation domain (AD) coding sequences in a modified version of plasmid pJG4-5 (Gyuris *et al.*, 1993) that contains the polylinker region from pEG202 (C. De Virgilio and D. DèMarini, unpublished), *S. cerevisiae TPS1*, *TPS2*, *TPS3*, and *TSL1* (accession numbers X61275, X70694, M88172,

and X72788, respectively) full-length coding sequences were amplified by PCR using Vent DNA polymerase (New England Biolabs) and either cloned genes (*TSL1* and *TPS3*) or genomic DNA (*TPS1* and *TPS2*) as templates. Appropriate restriction sites were introduced with the primers. The PCR products were cloned at the *Bam*HI site (*TPS1*), the *Not*I site (*TPS2*), the *Xho*I site (*TPS3*), or the *Eco*RI–*Sal*I site (*TSL1*) of pEG202. The constructs contain either 2 (EF for pEG202–*TSL1*), 5 (EFPGI for pEG202–*TPS1*), 12 (EFPGIRRPWRPQ for pEG202–*TPS2*), or 13 (EFPGIRRPWRPLE for pEG202-*TPS3*) additional amino acids between the LexA DBD and the first amino acid of the fused protein (M for all but Tps1; L, corresponding to the 10th residue, for Tps1). As a negative control, *MSB2* was fused to the DBD in pEG202 as described earlier (Simon *et al.*, 1995). For construction of a fusion of Tps1 to the AD in pJG4-5, the *TPS1* sequence was isolated from pEG202–*TPS1* by partial digestion with *Eco*RI and *Xho*I (*TPS1* has an internal *Eco*RI site) and cloned into the *Eco*RI site of pJG4-5.

To fuse Tps2 and Tps3 to the activation domain in pJG4-5, the defined sequences were amplified as above and cloned into the *Not*I site (for *TPS2*) of a pJG4-5 that had been modified to include a *Not*I site in its polylinker, or into the *Xho*I site of pJG4-5 (for *TPS3*). The Tsl1–AD fusion (*TSL1-1*) was originally isolated in a two-hybrid screen for proteins that interact with Tps2. The fused sequence in pJG4-5 codes for the amino acids 60–332 of the original Tsl1 sequence.

Strain EGY48 (Table 3) containing the *LexAop–lacZ* reporter plasmid pSH18-34 (Gyuris *et al.*, 1993) was cotransformed with pEG202 or a pEG202-derived plasmid expressing a LexA DBD fusion protein and with pJG4-5 or a pJG4-5-derived plasmid expressing an AD fusion protein. Beta-galactosidase activities were then assessed in three independent clones of each strain grown for 16 h at 27°C in minimal medium containing 2% (w/v) galactose, 1% (w/v) raffinose, and 20 μg leucine ml^{-1}.

Enzyme assays and determination of metabolite levels

Enzyme activities were measured in crude extracts made from cells harvested by centrifugation and washed once with distilled water. After resuspension in imidazole buffer (1 mM EDTA, 1 mM PMSF, 2 mM $MgCl_2$, 50 mM imidazole-HCl, pH 6.3), glass beads (0.5 mm diameter) were added and the cells were broken by four cycles of vortexing and cooling on ice, each for 1 min. The extracts were transferred into fresh tubes and centrifuged for 15 min at 20 000 × *g*. Supernatants were desalted on Sephadex G-25 columns (bed volume 2 ml) and then used for enzyme assays. All procedures were carried out at 4°C. Tre6*P* synthase activity was measured using the coupled assay described by Hottiger *et al.* (1987). The activity of Tre6*P* phosphatase was determined by incubation of extracts with Tre6*P* (Sigma) and quantification of the product trehalose by high-performance liquid chromatography (HPLC) analysis as described below (see also De Virgilio *et al.*, 1993).

For the determination of trehalose and Tre6*P*, 10 ml of exponentially growing cells was filtered (Whatman GF/C), washed three times with 5 ml of distilled H_2O, resuspended in 1 ml of H_2O and transferred to a boiling water bath for 10 min. After centrifugation (three times for 10 min at 20 000 × *g*, trehalose and Tre6*P* were determined in the supernatant by HPLC analysis as described by De Virgilio *et al.* (1993) using an anion-exchange column (CarboPac PA-1, Dionex) and a Dionex DX-300 Gradient Chromatography System. Peaks were detected with a pulsed amperometric detector (Dionex).

Protein concentrations in crude extracts were measured using the Bio-Rad protein assay according to the manufacturer's instructions, using BSA as the standard. Protein concentrations in the cultures were determined by means of a modified Lowry assay (Peterson, 1977), using BSA as the standard.

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CHAPTER II

IDENTIFICATION AND CHARACTERIZATION OF Rim15, A NEW PROTEIN KINASE INTERACTING WITH Tps1 IN A TWO-HYBRID SYSTEM

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INTRODUCTION

In the previous chapter it has been described that in *S. cerevisiae* trehalose is accumulated in response to a mild heat shock as well as upon stationary phase entry through the activity of the multimeric Tre6P synthase/phosphatase complex. This complex consists of four different subunits, encoded by the genes *TPS1* (coding for the Tre6P synthase), *TPS2* (coding for the Tre6P phosphatase), *TSL1* (coding for the 123 kDa putative regulatory subunit), and *TPS3* (coding for a *TSL1* homolog). It is by now widely accepted that accumulation of trehalose is advantageous for the survival of cells under different stress conditions. The regulation of trehalose synthesis however, has been the topic of a controversial debate (for a review see Thevelein, 1996). Moreover, interest in the regulation of Tps1 especially has been fueled by the discovery of its involvement in the control of glycolytic flux (see General Introduction and previous chapter, for a review see Thevelein and Hohmann, 1995). In the course of the debate, evidence for both transcriptional and posttranslational control of trehalose synthesis has been presented.

One argument for transcriptional control of both Tre6P synthase and Tre6P phosphatase by glucose repression stems from the observation that transfer of cells to growth medium without glucose led to an increase in the two enzyme activities which could be prevented by the addition of cycloheximide (François *et al.*, 1991). Hence, in response to the carbon source present, Tre6P synthase and Tre6P phosphatase might be subjected to control at the transcriptional and/or translational rather than at the posttranslational level. Recently, the transcription of *TPS1*, *TPS2*, *TPS3*, and *TSL1* has been studied in detail during exponential growth and in stationary phase. While *TPS1*, *TPS2*, and *TPS3* were more or less constitutively expressed, irrespective of growth phase and carbon source, abundance of *TSL1* was strongly repressed when cells were grown on glucose and completely derepressed during growth on a gluconeogenic carbon source or in stationary phase (Winderickx *et al.*, 1996). These findings may may not be easily reconciled with the idea that trehalose synthesis is controlled at the transcriptional level. However, since the relative abundance of a certain mRNA species may not reflect the actual amount of the corresponding protein, it is possible that *de novo* synthesis of the Tps proteins may be important for the regulation of trehalose accumulation. Therefore determination of the levels of Tps1, Tps2, Tps3, and Tsl1 protein should give a more precise picture of the importance of transcriptional and/or translational control mechanisms for the accumulation of trehalose during growth to stationary phase.

During a sublethal heat shock *S. cerevisiae* cells rapidly accumulate trehalose and this accumulation is mirrored by an increase in Tre6P synthase and to a lesser extent of Tre6P phosphatase activity *in vitro* (Hottiger *et al.*, 1987; De Virgilio *et al.*, 1993). Under

these conditions a strong but transient induction of transcription of both *TPS1* and *TPS2* was observed (Bell *et al.*, 1992; De Virgilio *et al.*, 1993). This expression pattern resembles the pattern described for members of the heat-shock protein family (Werner-Washburne *et al.*, 1989). The transcriptional induction of heat-induced genes is thought to be mediated by the heat-shock factor, a transcriptional activator constitutively bound to a *cis* promoter sequence, the heat-shock element (Sorger and Pelham, 1988). In the case of *TPS1*, *TPS2*, *TPS3* and *TSL1* it has recently been shown that their promoters contain several C_4T elements (Gounalaki and Thireos, 1994; Varela *et al.*, 1995). This cAMP-responsive element, also called STRE (for **s**tress **r**esponse **e**lement) has been identified as an important upstream regulatory element for the general stress response in *S. cerevisiae* (Kobayashi and McEntee, 1993; Marchler *et al.*, 1993), which is independent of heat-shock factor. In the case of *TPS2*, deletion of two of the four STREs present in the promoter indeed resulted in diminished stress induction of the gene (Gounalaki and Thireos, 1994). The other three genes, *TPS1*, *TPS3*, and *TSL1*, still await more detailed analysis. Taken together it seems that transcriptional induction of *TPS1* and *TPS2* and possibly also of *TPS3* and *TSL1* during a mild heat shock may at least partially contribute to heat-induced trehalose synthesis. In support of this assumption, it needs to be pointed out that addition of the translation inhibitor cycloheximide to cultures prior to a heat shock partially inhibited trehalose synthesis and the increase in Tre6P synthase activity (Hottiger, 1988; Neves and François, 1992). Also, it could be shown that trehalose synthesis is reduced at temperatures that are permissive for trehalose but non-permissive for protein synthesis in *S. cerevisiae* (De Virgilio *et al.*, 1991b). Taken together, these results indicate that at least during heat shock transcriptional activation and subsequent *de novo* synthesis of the Tre6P synthase as well as activation of the already present enzyme are responsible for the observed increase in trehalose synthesis. As in the case of stationary phase cells mentioned above, it still remains to be shown, however, that the heat-induced mRNAs of the *TPS* genes are also translated into the corresponding proteins under heat-shock conditions. Interestingly, the same type of investigations performed with the distantly related fungi *S. pombe* and *Neurospora crassa* revealed that in these organisms regulation at the transcriptional and/or translational level plays no role in trehalose accumulation during heat shock (De Virgilio *et al.*, 1990; Novente-Jordão *et al.*, 1996; Ribeiro *et al.*, 1997).

For the posttranslational control of trehalose synthesis several modes of action have been proposed, namely regulation by chemical or physical effectors of Tre6P synthase and Tre6P phosphatase, availability of substrates, or posttranslational modifications of Tre6P synthase, as will be presented in the following paragraphs.

The influence of a number of effectors was investigated, starting with the studies of Cabib and Leloir (1958), who provided the first data on the biochemical properties of the Tre6P synthase. They determined a pH-optimum of 6.6 in the presence of 25 mM Mg^{2+}.

Tre6P phosphatase was completely dependent upon the presence of Mg^{2+} (Vandercammen *et al.*, 1989). A powerful activator of the Tre6P synthase but not of Tre6P phosphatase is fructose-6-phosphate (Fru6P) (Londesborough and Vuorio, 1993). In the absence of Fru6P, phosphate at physiological concentrations (K_i about 5 mM) strongly inhibited Tre6P synthase activity whereas Tre6P phosphatase was stimulated (Vandercammen *et al.*, 1989; Londesborough and Vuorio, 1991; Londesborough and Vuorio, 1993). Several other enzymes involved in carbohydrate metabolism are also influenced by free phosphate (Banuelos *et al.*, 1977; François and Hers, 1988; François *et al.*, 1988) making it likely that the intracellular phosphate concentration could be an important regulator of carbohydrate metabolism. In this context it is interesting to recall that trehalose synthesis has been proposed to act as a kind of metabolic buffer system during exponential growth on glucose. Accordingly, low levels of free phosphate would promote trehalose synthesis, thereby enhancing the recovery of free phosphate from excessively formed glucose-6-phosphate (Glu6P), which would relieve a bottleneck in glycolysis (Thevelein and Hohmann, 1995).

Another potent effector of Tre6P synthase activity is the temperature. Inhibition of Tre6P synthase by phosphate and stimulation by Fru6P was temperature-dependent *in vitro*, both effects being most pronounced at 30°C, and negligible at 50°C. Tre6P phosphatase only displayed minor temperature dependency (Londesborough and Vuorio, 1993). Based on their findings that the increase in the activities of Tre6P synthase and Tre6P phosphatase observed at 40°C coincided with a strong decrease in trehalase activity at this temperature, François and Neves (1992) concluded that the induction of trehalose by heat shock was a consequence of temperature-dependent changes in the kinetic properties of the trehalose metabolic enzymes.

Kinetic studies of the Tre6P synthase revealed that the enzyme displays K_m values of 0.5 mM for UDP-glucose and 3.5 mM for Glu6P (Vandercammen *et al.*, 1989). While the K_m value of UDP-glucose is close to, the one of Glu6P is significantly higher than its intracellular concentration during growth on glucose (Gancedo and Gancedo, 1977). Therefore, it seems likely that the availability of Glu6P could be a determining factor in the biosynthesis of trehalose in yeast (Vandercammen *et al.*, 1989). In the case of the regulation of trehalose accumulation during heat shock, evidence for this interpretation was presented. In the initial phase of a mild heat shock the concentrations of the substrates for Tre6P synthase were found to rise 5-10 fold, prior to the activity increase of the enzyme. The increased availability of these substrates was attributed to a differential effect of temperature on glucose influx and glycolysis, causing upstream metabolites to accumulate (Winkler *et al.*, 1991; Ribeiro *et al.*, 1994). While the availability of substrates seems a plausible explanation for the observed accumulation of trehalose during heat shock, it remains to be shown how the same mechanism could be involved in the regulation of trehalose synthesis upon stationary phase entry.

Posttranslational modification of enzyme activity by means of phosphorylation is one possible way of modifying the activity of an enzyme directly. It has been shown that the trehalose-hydrolyzing enzyme neutral trehalase is activated by cAMP-dependent phosphorylation (Uno *et al.*, 1983; App and Holzer, 1989). It therefore seems reasonable to assume that trehalose synthesis could be regulated by the same pathway in an inverse manner. Indeed, Panek *et al.* (1987) claimed that Tre6P synthase could be inactivated by phosphorylation in a cAMP-dependent way. Their view has been challenged by Vandercammen *et al.* (1989) who criticized the enzyme assay used by the Panek group. Using a more reliable assay, Vandercammen *et al.* (1989) found no evidence for inactivation of Tre6P synthase or Tre6P phosphatase through cAMP-dependent phosphorylation either *in vivo* or *in vitro*.

Limited proteolysis might be another means of regulation of Tre6P synthase. During purification Tre6P synthase was observed to be partially degraded by endogenous proteolysis, resulting in its activation (Londesborough and Vuorio, 1991). From their results Londesborough and Vuorio (1991) deduced that the active site of Tre6P synthase could be partially occluded by a flap of protein from one end of the complex. A more detailed analysis revealed that the protein affected by proteolysis was Tsl1 and that this subunit was most probably responsible for holding together the intact trehalose synthase complex and conferring sensitivity to phosphate and Fru6P (Vuorio *et al.*, 1993). Activation by proteolysis *in vitro* was also reported from the Tre6P synthase of *Candida utilis* (Vicente-Soler *et al.*, 1991). Still, the role of proteolysis for the regulation of Tre6P synthase *in vivo* remains to be elucidated.

Finally, it has been shown that some sort of intrinsic regulation of Tre6P activity by one or both of the large subunits, Tsl1 and Tps3, respectively, exists *in vivo*. Trehalose accumulation in heat shock as well as in stationary phase depends upon the presence of either Tps3 or Tsl1 (Reinders *et al.*, 1997; Bell *et al.*, 1998). Deletion of *TPS3* was found to have no effect upon the induction of Tre6P synthase activity in cells growing to stationary phase, whereas deletion of *TSL1* prevented the induction (Ferreira *et al.*, 1996; Bell *et al.*, 1998). This is in good agreement with the observed expression levels of *TPS3* and *TSL1* under different growth conditions (see above, Winderickx *et al.*, 1996) and could be interpreted in such a way that the two subunits are responsible for control of trehalose synthesis under different conditions. During heat shock, however, no different influence of the loss of *TSL1* or *TPS3* could be detected (Reinders *et al.*, 1997).

From the results obtained so far it seems likely that both transcriptional and posttranslational regulatory mechanisms are involved in trehalose synthesis. In this context, it is interesting to note that in *Aspergillus niger*, a filamentous fungus that possesses two Tre6P synthases, both are differentially regulated. While one is constitutively expressed, the other is specifically induced during heat shock (Wolschek and Kubicek, 1997). In *S. cerevisiae* one enzyme is responsible for trehalose synthesis under all conditions. There-

fore it may be speculated that the nature of the control mechanisms of Tre6P synthase (*i.e.* the induction or repression of *TPS1* expression and posttranslational activation of Tps1) depends on the physiological conditions.

In view of the controversy over the mode of control of trehalose synthesis and especially in view of the proposed role of Tps1 as a possible control element of sugar influx into glycolysis we decided to perform a two-hybrid screen for proteins which interact with Tps1. Such a screen was expected to yield (i) regulatory proteins of Tps1, (ii) unidentified subunits of the Tre6P synthase/phosphatase complex, and (iii) elements of the glycolytic pathway that interact with Tps1. This screen was initiated by C. De Virgilio and continued by C. Funk with the technical assistance of N. Bürckert. A second screen using the second enzymatic entity of the Tre6P synthase/phosphatase complex, Tps2, as bait followed. A first transformation was done by C. Funk and the screen was then continued by myself with the technical support of N. Bürckert. This chapter of my thesis summarizes the results of these two screens and then mainly focuses on the characterization of a new protein kinase gene, *RIM15*, that was identified as an interactor of Tps1. The putative role of Rim15 in trehalose synthesis and nutrient signaling was investigated.

RESULTS

Two-hybrid screens with Tps1 and Tps2

A two-hybrid screen was performed to identify proteins that interacted with Tps1 and a second screen to identify proteins that interacted with Tps2. This study was undertaken for three reasons. First, while four putative subunits of the Tre6P synthase/phosphatase complex had been identified (Bell *et al.*, 1992, Manning *et al.*, 1992, De Virgilio *et al.*, 1993, Vuorio *et al.*, 1993), it still remained to be shown that they actually form a complex *in vivo* (see Chapter I). Second, regulation of trehalose synthesis by posttranslational mechanisms had been discussed (see Introduction) but no interacting regulatory protein had been isolated. Third, especially in view of the role of Tps1 as a putative regulator of glycolysis and/or sugar uptake, it would have been interesting to identify interacting proteins involved in these functions (*e.g.* hexokinases, glycolytic enzymes, hexose transporters).

A genomic DNA library from yeast was screened using the LexA two-hybrid system ("interaction trap", Zervos *et al.*, 1993). Selection was performed with strain EGY48 that contained pEG202-*TPS1* (expressing DBD-Tps1) or pEG202-*TPS2* (expressing DBD-Tps2) and also pSH18-34, a highly sensitive LexAop-lacZ reporter (Gyuris *et al.*, 1993). A pool of cells containing 7.5×10^5 primary library transformants was plated onto SD plates containing galactose but no leucine and the first 72 (DBD-Tps1) or 104 (DBD-Tps2) colonies that appeared were picked. These were further analyzed for unambiguous galactose-dependent blue color on X-gal medium. The remaining colonies were then checked by restriction analysis and partial sequencing (see Material and Methods) and assigned to 38 (DBD-Tps1) or 33 (DBD-Tps2) classes. From these classes a vast number could be discarded after sequence comparison with the EMBL data base because they were found to encode known transcription factors and other false positives encountered in previous two-hybrid screens by other groups (Claudio De Virgilio, personal communication), or because they contained non-coding sequence. Plasmids isolated from each remaining class of positive clones were backtransformed into strain EGY48 and their β-galactosidase activity on liquid galactose-containing medium determined. Eventually three classes of interactors with Tps1 and two classes of interactors with Tps2 remained. They are listed in Table 1. Partial sequencing of the first class of Tps1 interactors showed it to be a previously unidentified gene with homology to protein kinases. Consequently, it was called *TAK1* (for **T**ps1 **a**ssociated protein **k**inase) and because a role in the regulation of trehalose synthesis was anticipated it was decided to commence detailed analyses with this gene. As explained in Material and Methods and in the Introduction to this chapter,

the nomenclature suggested by Vidan and Mitchell (1997), who called the same gene *RIM15* and the gene product Rim15, was later adopted because their publication was the first showing a function of Tak1/Rim15 (see below). The other two classes of Tps1 interactors showed no obvious homology to any sequence at the EMBL data base at that time. In the meantime however, the corresponding full length genes have been sequenced by the *S. cerevisiae* genome project. They were assigned the names YPL032C (accession number U44030) and YBR061C (accession number Z35930). Both genes have no known function. YPL032C is highly homologous to *PAM1* (Hu and Ronne, 1994; 42% identity over the entire predicted amino acid sequence). *PAM1* was identified in a screen for multicopy suppressors of protein phosphatase 2A (PP2A) deficiency. Strong overexpression of *PAM1* led to growth arrest and a filamentous phenotype. It was speculated that *PAM1* might encode a regulatory element that either acts on one of the systems regulated by PP2A or by counteracting the activity of a protein kinase (Hu and Ronne, 1994). PP2A has been shown to be involved in regulating various metabolic processes. In budding yeast it is a regulator of cell division and morphogenesis and has been found to inhibit the entry of cells into mitosis. In mammalian systems PP2A is known to dephosphorylate downstream targets for cAPK (for reviews see Cohen, 1989; Shenolikar, 1994). The fact that the same screen that yielded a gene for a new protein kinase (*RIM15*) also yielded the gene for a putative kinase-inhibiting protein, makes it seem worthwhile to further investigate the role of YPL032C in the future. Recently YPL032C has also been entered into the data base under the name of *SVL3* (styryl dye vacuolar localization) but no further information has been published so far (Zheng *et al.*, 1997). YBR061C has some homology to the *ftsJ* protein from *E. coli* (Tomoyasu *et al.*, 1993, 37.6% identity over the entire predicted amino acid sequence) but the significance of this homology remains unclear at the moment. Disruption of *ftlJ* in *E. coli* causes slow growth and formation of filaments (Tomoyasu *et al.*, 1993) but apart from these observations no function has been assigned to the FtsJ protein.

None of the subunits of the Tre6P synthase/phosphatase complex were isolated in the screen with DBD-Tps1 which is somewhat surprising since in a directed two-hybrid assay it has been demonstrated that Tps1 can interact with Tps1, Tps2, Tps3 and Tsl1 (Reinders *et al.*, 1997; see Chapter I of this thesis). It seems that the screen was not fully saturated even though care had been taken to ensure this by transforming an appropriate number of cells. This explanation is supported by the fact that the same *RIM15* library plasmid was identified nine times in the screen while the other two genes were found only once.

The screen for interactors of Tps2 yielded two classes of interactors. One of them was Tsl1, one of the large subunits of the Tre6P synthase/phosphatase complex (Vuorio *et al.*, 1993; Reinders *et al.*, 1997). The corresponding library plasmid was found ten

times in the screen. The second interactor YHR202W (accession number U00030) again showed no homologies to any other gene in the EMBL data base.

Table 1: *Results of two-hybrid screens using Tps1 and Tps2 as baits*

DBD-fusions	Identity of AD fusions	β-Galactosidase activity	Amino acid residues	References
TPS1	*TAK1 / RIM15 / YFL033C*	44	761-1051	Murakami *et al.*, 1995; Vidan and Mitchell, 1997; Reinders *et al.*, 1998
TPS1	*YPL032C / SVL3*	213	3-394	Bussey *et al.*, 1997, Zheng *et al.*, 1997
TPS1	*YBR061C*	129	774-815	
TPS2	*TSL1*	344	60-332	Vuorio *et al.*, 1993
TPS2	*YHR202W*	490	43-110	

β-Galactosidase activities in Miller units of negative controls (with pEG202, DBD-Msb2) were subtracted from the activities displayed in the table (values were below 4 units for all controls). *YPL032C*, *YBR061C*, and *YHR202W* were identified once, *RIM15* nine times, and *TSL1* ten times.

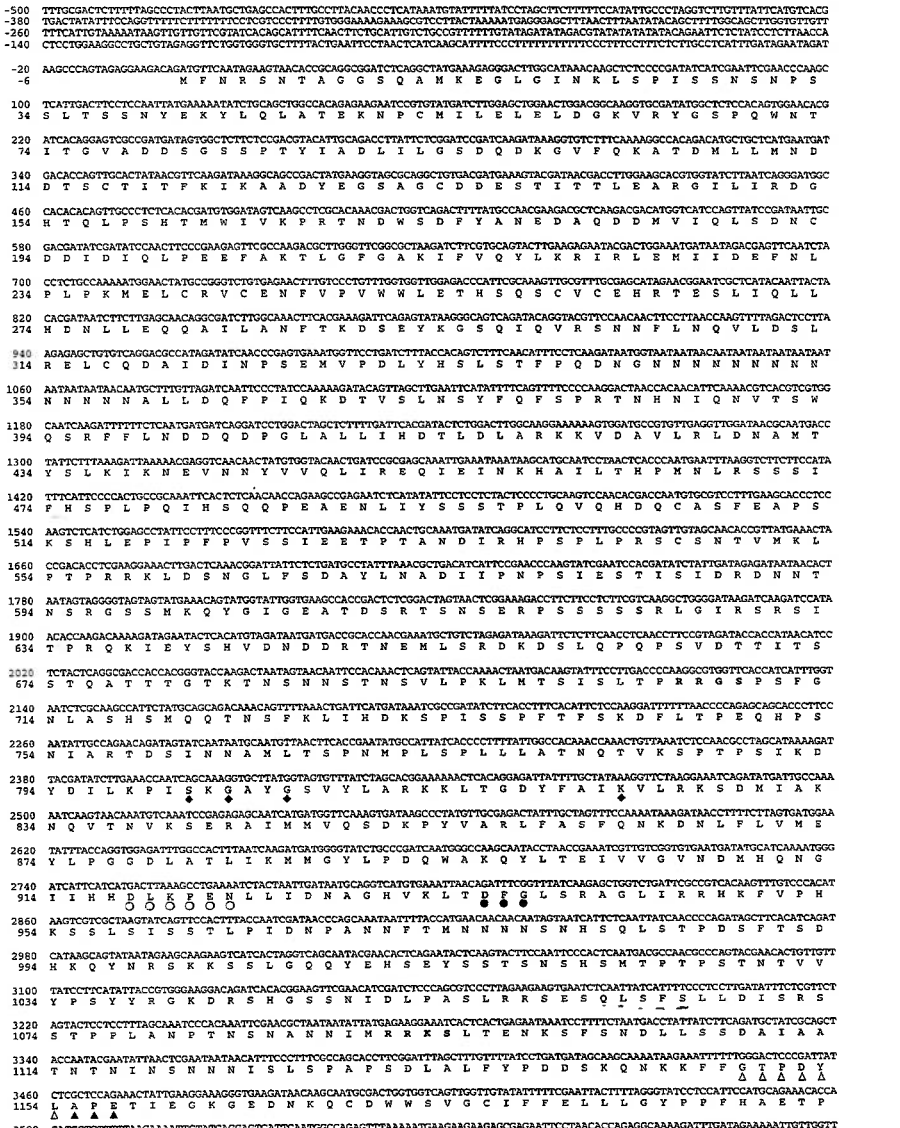
In a second round of assays all of the identified interactors listed in Table 4 were analyzed for the specificity of their interaction with the bait used. This was done by testing the ability of AD-Rim15, AD-YPL032C, AD-YBR061C, AD-Tsl1, and AD-YHR202W (the previously isolated library plasmids were used) to interact with DBD-Tps1, DBD-Tps2, DBD-Tps3, DBD-Tsl1, DBD-Msb2, and the empty vector pEG202 (see Reinders *et al.*, 1997, Chapter I of this thesis, for a detailed description of the DBD-fusions). β-Galactosidase activity was assayed. AD-Rim15, AD-YPL032C and AD-YBR061C were shown to interact only with the bait originally used to isolate them. Since Tps1, Tps2, Tps3, and Tsl1 share high homology over the entire stretch of Tps1 it can therefore be concluded that the interactions observed are highly specific. AD-YHR202W interacted with three of the four subunits of the Tre6P synthase/phosphatase complex (DBD-Tps1: 272 Miller units; DBD-Tps3: 73 Miller units) but not with the fourth (DBD-Tsl1) and not with the unrelated control (DBD-Msb2) or the empty vector. It can therefore with caution also be considered a specific interactor. AD-Tsl1 also interacted with DBD-

Tps1, but not with DBD-Tps3 or with the unrelated control. Since these data are also further supported by physiological experiments, it can be concluded that the two-hybrid results for Tsl1 again reflect specific interactions (Reinders *et al.*, 1997).

Cloning and sequence analysis of *RIM15*

Sequence comparison of the insert of pJG4-5-*RIM15* had shown it to have high homology to protein kinases. In order to identify the full length gene a genomic library in pSEY8 was screened and several clones were obtained. The longest clone (#8, about 9 kb) was sequenced and found to contain an open reading frame of 3.3 kb, predicted to code for a protein of 122 kDa. Because the sequence upstream of the first methionine contained several in-frame stop codons this was considered to be the start of the ORF. Sequence comparison revealed homology to members of the family of serine/threonine protein kinases. The gene was therefore originally designated *TAK1* (for Tps1 associated protein kinase) and its gene product Tak1. During the course of this study, the same gene was also sequenced by the *S. cerevisiae* genome project and assigned the name YFL033C (accession numbers D50617/D44605). Recently the same gene was also isolated in a screen for mutations resulting in reduced expression of *IME2*, a gene induced in early meiosis (Vidan and Mitchell, 1997). Accordingly it was called *RIM15* (for reduced expression of *IME2*) and it was proposed to have a role as an activator of early meiotic genes. As already explained before, this name will be used in this thesis instead of *TAK1*. The complete sequence of YFL033C/*RIM15* was shown to be 5.3 kb instead of 3.3 kb. The difference in length between our sequence and the sequence of YFL033C/*RIM15* was due to a faulty library clone. The pSEY8 clone used by us for sequencing *RIM15* was a hybrid clone and what we considered to be promoter sequence of *RIM15* was then found to belong to another chromosome. Figure 2 (Material and Methods) gives an overview of the *RIM15* gene, indicating length and position of the initially identified LexA clone and the position of the first identified start codon (arrow). Eventually 6316 bp were sequenced from phage clone ATCC 70791 (from ATCC, Rockville, MD, USA), containing the whole 5313 bp *RIM15* gene. This gene is predicted to code for a protein of 1770 amino acids or approximately 197 kDa. The clone isolated in the two-hybrid study corresponds to amino acids 761-1051 of Rim15, this part of the sequence includes kinase subdomains I-VII. The sequence was submitted to the EMBL data base under the name of *TAK1* and given the accession number AJ001030.

The predicted Rim15 protein bears strong homology to known protein kinases (Figure 1 and 2). All eleven major conserved kinase subdomains (Hanks *et al.*, 1988)



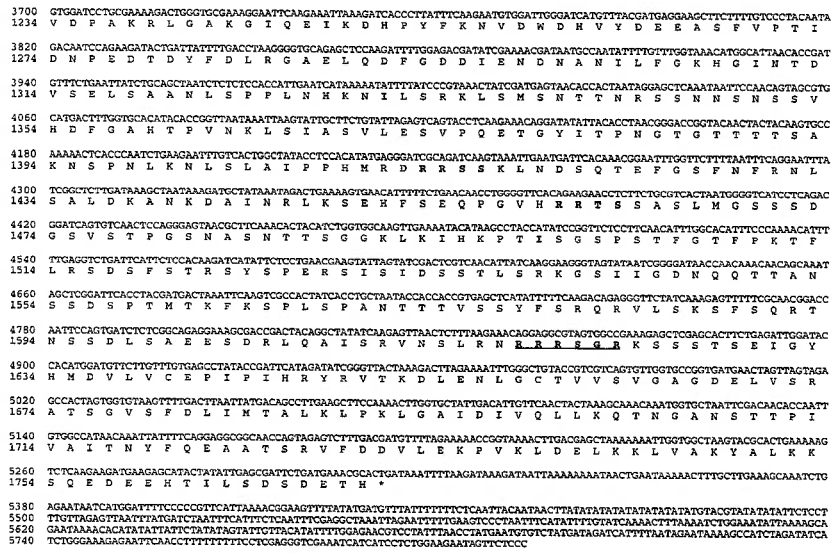


Figure 1: Nucleotide sequence and deduced amino acid sequence of *RIM15*. In addition to the coding sequence a total of 1000 nucleotides of up- and downstream sequence are shown. The ATP-binding site is marked with diamonds. Invariant sequences among known protein kinases are marked with closed circles (Asp-Phe-Gly) or closed triangles (Ala-Pro-Glu), consensus sites typical for serine/threonine protein kinases are marked with open circles (Asp-Leu-Lys-Pro-Glu-Asn) or open triangles (Gly-Thr-X-X-Tyr-X). Consensus sequences for cAPK phosphorylation and for protein kinase C phosphorylation are shown in bold and in bold, underlined letters, respectively. The stop codon is indicated by an asterisk.

were found. The consensus Gly-X-Gly-X-X-Gly in domain I followed by an invariant Lys 7-16 amino acids later, also found in many nucleotide binding proteins, is thought to be part of the ATP-binding site (Kamps *et al.*, 1984; Hannink and Donoghue, 1985; Hanks *et al.*, 1988; Saraste *et al.*, 1990). The corresponding sequence in the *RIM15* gene is marked in Figure 1 with diamonds. The most highly conserved stretches in the catalytic domains are Asp-Phe-Gly (in subdomain VII, indicated by closed circles in Figure 1) and Ala-Pro-Glu (in subdomain VIII, indicated by closed triangles in Figure 1). Subdomains VII and VIII are also known to contain residues that are specific to either serine/threonine or tyrosine kinases. They might play a role in recognition of the correct hydroxiamino acid (Hanks *et al.*, 1988). The sites specific for serine/threonine phosphorylation are Asp-Leu-Lys-Pro-Glu-Asn, in subdomain VII (marked with open circles in Figure 1) and a less strongly conserved region in subdomain VIII, immediately on the amino-terminal side of the Ala-Pro-Glu consensus, is Gly-Thr/Ser-X-X-Tyr/Phe-X-Ala-Pro-Glu (marked with open triangles in Figure 1). Accordingly, a FASTA/TFASTA search at the EMBL data base placed the Rim15 protein in the family of serine/threonine protein kinases, with especially high homologies to members of the subfamily of cAMP-dependent protein kinases (see Figure 2). This was confirmed by the recently published dendrogram of the *S. cerevisiae* protein kinase superfamily that places Rim15 (YFL033C) in the AGC group on a branch close to the cAMP-dependent protein kinases (Hunter and Plowman, 1997).

The predicted amino acid sequence of *RIM15* has some intriguing features. One feature is the presence of extended amino- and carboxy-terminal domains that are distinct from the conserved kinase domain of the protein. These parts of Rim15 have no homology to any other *S. cerevisiae* gene in the EMBL data base. The same is true for the uncommon additional stretch of 210 amino acids between the conserved kinase domains VII and VIII (Hanks *et al.*, 1988). The closest known homolog of *RIM15* is the *Schizosaccharomyces pombe cek1*$^+$ which was isolated as a multicopy suppressor gene for the temperature sensitive defect in progression through mitotic anaphase caused by *cut8-563* (Samejima and Yanagida, 1994). Interestingly, these two genes also share homologies in their extended amino-terminal domains upstream of the putative ATP-binding sites (26.2% identity between the 800 and 595 amino-terminal amino acids of Rim15 and Cek1, respectively) and Cek1 also contains an insertion of 112 amino acids between kinase domains VII and VIII. In view of a possible regulation of Rim15 function the presence of four consensus sites for cAPK-dependent phosphorylation, Arg-Arg-X-Ser (Edelman *et al.*, 1987; printed in bold, Figure 1) and one consensus site for protein kinase C dependent phosphorylation (Arg-X-X-Ser-X-Arg, Kemp and Pearson, 1990; bold and underlined, Figure 1) are worth noting.

```
Rim15   PSIkDYdILKPISKGAyGSVYLARKKlTGDYFAIKVLrKSdMIAKNQVtNV   839
Cek1    PSInDYkILKPISKGAFGSVYLAqKrtTGDYFAIKiLKKSnMIAKNQViNV   634
Tpk1    YSlQDFqILRtLGtGsFGrVhLiRsrhnGRYYAMKVLKKeivVrlkQVeHT   132
Sch9    YgpQDFevLRlLGKGtFGqVYqvkKKdTqRiYAMKVLsKkviVkKNeiqHT   457

Rim15   ksERAIMMvQsdK..PyVARLfasFQnKDnLFLVMEYlpGGDLatLiKmMG   889
Cek1    raERAILMSQges..PFVAkLyyTFQsKDyLYLVMEYlnGGDcgSLLKtMG   684
Tpk1    ndER.lMlSivth..PFIiRmwgTFQdaqqiFmiMDYieGGELFSLLrKsq   181
Sch9    igERnILyttasKssPFIvgLkfsFQtptdLYLVtDYmsGGELFwhLqKeG   509

Rim15   yLPdqWAKqYltEiVvgvnDmHqNGIIHhDLKPENLLIDnaGHvKLTDFGL   939
Cek1    vLdlDWirtYIAEtVLcLgDLHDrGIIHRDiKPENLLIsqNGHlKLTDFGL   734
Tpk1    RFPnpvAKFYaAEvcLALEyLHskDIIYRDLKPENILLDkNGHIKiTDFGf   231
Sch9    RFseDrAKFYIAElVLALEhLHDNDIvYrDLKPENILLDaNGnIalcDFGL   559

Rim15   -{ 210} - GTPDYLAPEtIeGKgedNKqcDWWSvGCifFElLlGYPPFhAE  1190
Cek1    -{ 124} - GTPDYIAPEVILGnPGi.KasDWWSLGCvvFEfLfGYPPFnAE   898
Tpk1    -{  12} - GTPDYIAPEVvstKP.YNKsiDWWSfGiLiyEMLaGYtPFyds   284
Sch9    -{  13} - GTteYLAPEllLdetGYtKmvDfWSLGvLiFEMccGwsPFfAE   614

Rim15   TPDaVFkKILsGvIqWPeFkNEeEeRefLtpEAKDLIekLLvvDPAkRLG.  1240
Cek1    TPDqVFQnILarrInWPa.....EvftaeSsvAlDLIdRLLcmNPAnRLG.   944
Tpk1    NtmKtYeKILnaelrFPpFfNE.........dvKDLlsRLitRDlsqRLGn   325
Sch9    NnqKmYQKIafGkvkFP........RdvLSqEgrsfvkgLLnRNPkhRLG.   556

Rim15   .A.kGiqEIKdHPyFKnVdWDhvy..dEE..asFVPtIdnPE.DTdYFDlR  1285
Cek1    .A.NGvEEIKAHPFFKsVnWDtiL..eEd..PPFVPkpfSPE.DTvYFDsR   989
Tpk1    lq.NGtEdvKnHPwFKeVvWEkLLsrniE..tPyePpIqqgqgDTSqFDky   375
Sch9    .AiddgrElrAHPFFadidwEaLk..qkkipPPFkPhlvSet.DTS.....   699
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Figure 2: Sequence comparison of the catalytic domains of *S. cerevisiae* Rim15, *S. pombe* Cek1, *S. cerevisiae* Tpk1 and *S. cerevisiae* Sch9. Tpk1 (Toda *et al.*, 1987b) and the highly homologous Sch9 (Toda *et al.*, 1988) are members of the subfamily of cAMP-dependent protein kinases in *S. cerevisiae*. Amino acids which are the same between at least two of the four sequences are shown in capital letters, those which are the same in at least three of the four sequences are shown in bold capital letters. Dots indicate gaps inserted to maximize sequence alignment. In brackets are the numbers of amino acids inserted at this site.

Gene disruption of *RIM15*

In order to examine the effect of loss of *RIM15*, a one-step gene disruption in the haploid RH144-3A wild-type background was carried out (see Material and Methods, Figure 2). Two clones (#2, #10) growing in the absence of uracil were picked and the integration of the 2.3 kb *Cla*I-*Cla*I fragment containing *URA3* inserted into the kinase domain of the *RIM15* locus was confirmed by Southern blot analysis (Figure 3). Both clones analyzed carried the *RIM15* disruption. For all following experiments clone #2 was chosen and designated AR3. Since the disruption mutant was viable it could be concluded that *RIM15* is not essential.

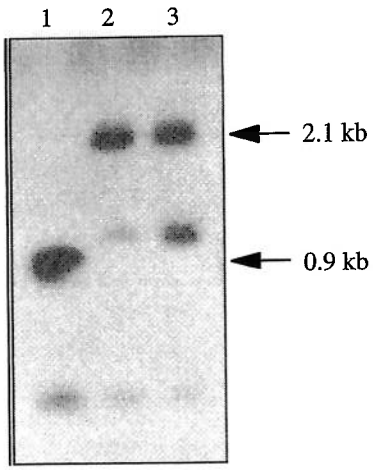

Figure 3: Southern blot of yeast genomic DNA. The DNA was digested with *Cla*I and analyzed by Southern blotting using the internal 0.9 kb *Cla*I-*Cla*I fragment of *RIM15* as probe. Lane 1: RH144-3A (wild type); lane 2: AR3, clone #2; lane 3: AR3, clone #10. Molecular weights, determined using λ *Hind*III as marker, are indicated on the right. AR3, clone #2 was used for all following experiments.

Effects of *RIM15* disruption

A strain with a disrupted RIM15 *does not display a phenotype during logarithmic growth phase*

The *RIM15* disruption strain AR3 was examined during exponential growth and its phenotype compared with the phenotype of the corresponding wild-type strain RH144-3A. Initially, cells were checked for their ability to grow on different carbon sources (Table 2). Cultures were grown on SD media supplemented with either glucose, fructose, sucrose, maltose, galactose, or glycerol, and the appropriate amino acid requirements. The disruption strain grew on the same carbon sources as the wild-type strain with similar growth rates. In general both strains grew better on rapidly fermentable carbon sources such as glucose, fructose and sucrose and less well on maltose, galactose or glycerol. The latter three carbon sources had to be supplemented with a small amount of glucose (0.5% [w/v]) in order to initiate growth. No morphological differences were found during exponential phase between the *rim15::URA3* strain and the wild type when cells were examined under the microscope.

Table 2: *Growth rates on different carbon sources*

	Glucose	Fructose	Sucrose	Maltose	Galactose	Glycerol
RIM15	0.233	0.229	0.240	0.182	0.180	0.178
rim15::URA3	0.233	0.215	0.239	0.172	0.182	0.174

Growth rates were determined during exponential growth on SD medium containing either 2% (w/v) (glucose, fructose, sucrose, maltose, galactose) or 3% (w/v) (glycerol) of carbon source. In the case of maltose, galactose and glycerol 0.5% (w/v) of glucose were added to the media in order to initialize growth. The numbers represent the slopes of the semi-logarithmic growth curves (h^{-1}).

Because *RIM15* had been found as an interactor of *TPS1* in the two-hybrid system, its possible role in trehalose metabolism was investigated. The activities of the trehalose synthesizing enzymes Tre6P synthase and Tre6P phosphatase and of the trehalose hydrolyzing enzyme neutral trehalase were assayed in log-phase and during heat shock (1 h at 42°C), as well as the amounts of trehalose accumulated under these conditions. The results are summarized in Table 3. Both strains, wild type and *rim15*-mutant, did not differ significantly from each other regarding the parameters tested, even though the *rim15::URA3* strain showed somewhat lower Tre6P synthase activities and trehalose content during heat shock in most experiments. In parallel with the enzyme activities and trehalose levels, thermotolerance, that is the ability to survive a challenging heat shock, was tested. Strains were incubated at 51°C for 8 min, either after growth at 27°C or following the 1-h preconditioning heat shock at 42°C. Both wild type and *rim15*-mutant survived equally well. The cells not subjected to the preconditioning treatment were very heat sensitive in both strains, while 50% of the preconditioned cells of both strains survived the challenging heat shock (Table 3).

Trehalase activity was also studied in a temperature shift experiment. It had been shown that the trehalose-degrading enzyme neutral trehalase is rapidly activated in cultures that are shifted from 40°C to 27°C (De Virgilio *et al.*, 1991a). When this experiment was performed with strains RH144-3A and AR3, the induction levels were similar in both strains (Table 3). As has been described in the Introduction, this activation of trehalase is mediated by phosphorylation in a cAMP-dependent manner, probably by cAPK, the key enzyme of the Ras/cAMP pathway. The fact that the activation pattern was the same for both strains, would suggest that Rim15 is not part of the signaling chain leading to trehalase activation after temperature downshift.

As part of the study of the role of Rim15 it was also determined whether the expression levels of *RIM15* were induced in wild-type cells as a response to different forms

of physiological stress, such as heat shock, osmotic shock, methanol stress and oxidative stress. This seemed interesting because the ability to survive certain forms of physiological stress has often been correlated with the ability to accumulate trehalose and because Rim15, as a protein kinase and as an interactor of Tps1 in the two-hybrid system, could be important for the regulation of trehalose synthesis under stress conditions.

Table 3: *Phenotype of* rim15::URA3 *during log-phase and heat shock*[1]

	RIM15		*rim15::URA3*	
	Log-phase	Heat shock[2]	Log-phase	Heat shock[2]
Enzymes and metabolites				
Tre6P synthase[3]	0.231 ± 0.160	2.011 ± 0.477	0.182 ± 0.174	1.904 ± 0.545
Tre6P phosphatase[3]	0.059 ± 0.024	0.158 ± 0.050	0.049 ± 0.010	0.108 ± 0.025
Trehalase[3]	0.190 ± 0.085	0.170 ± 0.010	0.190 ± 0.099	0.157 ± 0.049
Trehalose[4]	0.038 ± 0.033	0.260 ± 0.051	0.037 ± 0.035	0.226 ± 0.084
Thermotolerance[5]	0.002 ± 0.002	51.17 ± 16.06	0.002 ± 0.002	46.58 ± 12.34

1 Strains used were RH144-3A (*RIM15*) and AR3 (*rim15::URA3*). Results shown are the mean ± standard deviation of at least three experiments.
2 Exponentially growing cultures were heat shocked for 1 h at 42°C.
3 Enzyme activities are given in µkat/g protein.
4 Trehalose concentration is given in g/g protein.
5 Thermotolerance was determined as the percentage of survival after subjecting the cells to an 8 min challenging heat shock at 51°C, either without (log-phase) or with (heat shock) preconditioning for 1 h at 42°C.

RNA was prepared from cells subjected to these stress treatments and used in Northern blot analysis. The blots were hybridized with either a *RIM15* probe or a *TPS2* probe as a control (Figure 4). *TPS2* was used as a control because its expression has been shown to be induced during heat and methanol stress (De Virgilio *et al.*, 1993; Vahlensieck, 1996). As can been seen from the autoradiograms in Figure 6, *TPS2* was lowly expressed during log-phase. However, it became strongly and transiently induced during heat shock, and, albeit to a lower extent, also during the other stress conditions used. This is in agreement with earlier results of De Virgilio *et al.* (1993) who had

demonstrated that Tps2 is a heat-shock protein. The induction due to methanol stress was extremely rapid and can already be seen in the time zero sample that was taken immediately after addition of the agent. The time needed for sample preparation was sufficient to lead to a much stronger signal than in all the other treatments at time zero. In order to circumvent this problem the time zero sample should generally be taken before the stress condition is imposed. The signal in cells treated with oxidative stress was rather weak in general. Most probably this was due to a rapid loss of viability during the stress treatment. When survival was tested by plating the cells at the end of the experiment on YPD agar and counting colonies after two days (100% = non-stressed cells), only about 50% of the cells stressed with H_2O_2 had survived, compared with 80% of the methanol stressed cells and 100% of the osmotic shock and heat shock treated cells. From the blots probed with *RIM15* it can be concluded that *RIM15* is constitutively expressed during log-phase and that its expression does not increase due to any of the stress conditions tested.

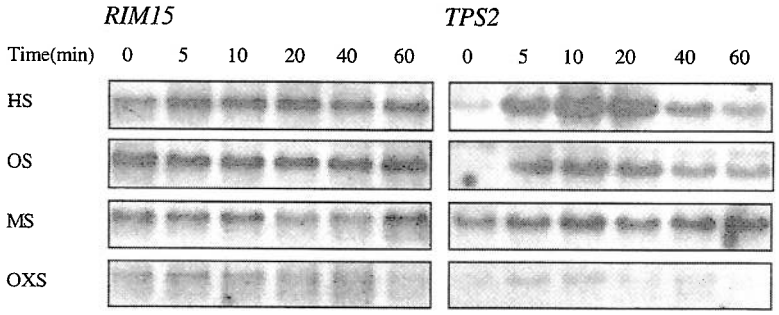

Figure 4: Effect of different stress treatments on the expression levels of *RIM15* and *TPS2*. Total RNA was prepared from log-phase cells grown at 27°C on full medium and subjected to different stress treatments. Samples were taken at the times indicated. The blots were hybridized with either a *RIM15* or a *TPS2* DNA probe. The stress conditions used were (i) heat shock (HS): shifting cells from 27°C to 42°C, (ii) osmotic shock (OS): 0.3 M NaCl, (iii) methanol stress (MS): 10% (v/v) methanol, and (iv) oxidative stress (OXS): 0.3 mM H_2O_2.

Summarizing the results obtained with log-phase cultures it can be concluded that the deletion of *RIM15* does not lead to any obvious phenotype neither in exponentially growing nor in cells subjected to physiological stress while growing on glucose.

Effects of disruption of RIM15 *in stationary phase include defects in the accumulation of trehalose and glycogen and starvation sensitivity*

While disruption of *RIM15* had no detectable consequences in exponential phase, the same cells showed a distinct phenotype in stationary phase. First, examination under the microscope revealed that the cells of the disruption strain AR3 were somewhat smaller and often had buds even in stationary phase. However, cell number (counting budded cells as *one* rather than as *two* cells), dry weight and protein content of the two strains compared were the same (Table 4). The enzyme activities of Tre6P synthase, Tre6P

Table 4: *Phenotype of* rim15::URA3 *in stationary phase*[1]

	RIM15	*rim15::URA3*
Growth		
Cell number (cells/ml)	$1.44*10^8 \pm 1.25*10^7$	$1.38*10^8 \pm 9.54*10^6$
Dry weight (mg/ml)	3.07 ± 0.03	2.7 ± 0.00
Protein content (mg/ml)	0.777 ± 0.038	0.780 ± 0.074
Enzymes		
Tre6P synthase (µkat/g protein)	0.889 ± 0.042	0.905 ± 0.046
Tre6P phosphatase (µkat/g protein)	0.131 ± 0.018	0.157 ± 0.023
Trehalase (µkat/g protein)	0.064 ± 0.018	0.082 ± 0.024
Metabolites		
Trehalose (g/g protein)	0.259 ± 0.011	0.067 ± 0.001
Glycogen (g/g protein)	0.077 ± 0.001	0.030 ± 0.021
Survival (%)[2]	108.6 ± 10.3	3.3 ± 0.8

[1] Cells were grown to stationary phase on SD medium containing 1% (w/v) of glucose. If not stated otherwise samples were taken after 3 d. Strains used were RH144-3A (*RIM15*) and AR3 (*rim15::URA3*). Results shown are the mean ± standard deviation of at least three experiments.

[2] Percentage of survival after 6 d. Cells were plated on YPD agar and colonies counted after incubation at 27°C for 3 d. The number of colonies was compared to the total number of cells counted in the culture.

phosphatase and trehalase again showed no significant differences between wild-type and mutant strain. Remarkably, the *rim15::URA3* strain only accumulated about 25% of the amount of trehalose the wild type did (0.067 g/g protein in the mutant, compared with 0.259 g/g protein in the wild type). The other carbohydrate besides trehalose that is found in considerable amounts in stationary yeast cells is the storage carbohydrate glycogen. The determination of glycogen content in the mutant and the wild type showed that loss of *RIM15* also has an effect on the glycogen level. The mutant contained about 60% less glycogen (0.030 g/g protein) than the wild-type strain (0.077 g/g protein).

Because *rim15::URA3* cells took notably longer to enter exponential phase than the wild type, but then grew with the same growth rate (see Table 5), it was tested whether loss of *RIM15* had any effect on the survival of the cells. Stationary cultures (6 d) of RH144-3A and AR3 were appropriately diluted and plated onto YPD agar. The number of cells/ml was counted with a hematocytometer (=100%). After 3 d the colonies formed on the agar plates were counted and compared with the number of cells in the cultures. While 100% of the wild-type cells survived, only 3.3% of the mutant cells did.

It can be concluded that disruption of *RIM15* not only leads to defects in the accumulation of trehalose and glycogen in stationary phase, but also renders the cells very sensitive to starvation.

Deletion of *RIM15*

At this point in the project the question arose whether a disruption of *RIM15* was really sufficient to study the role of its gene product Rim15. It could have been possible that the fairly large parts of the putative protein kinase outside of the immediate kinase domains (see Material and Methods, Figure 2) had some additional functions that would not have been affected by the disruption. Therefore, it was decided that a complete deletion of *RIM15* would be made in another wild-type background. The diploid wild-type strain YEF473 was chosen for this purpose because it had a range of suitable auxotrophic markers available (see Material and Methods, Table 1).

A one-step PCR-based gene deletion method was used to replace the complete *RIM15* ORF by the *kanMX2* marker, coding for geneticin resistance (see Material and Methods, Figure 3A). The 1.56 kb PCR-derived deletion fragment was used to transform the diploid wild-type strain YEF73. Transformants were selected by their ability to grow in the presence of geneticin and checked by PCR for the correct integration of the marker at the *RIM15* locus (see Material and Methods, Figure 3B). One resulting correct transformant (AR1) was sporulated, and tetrad dissection was carried out. The *RIM15* deletion

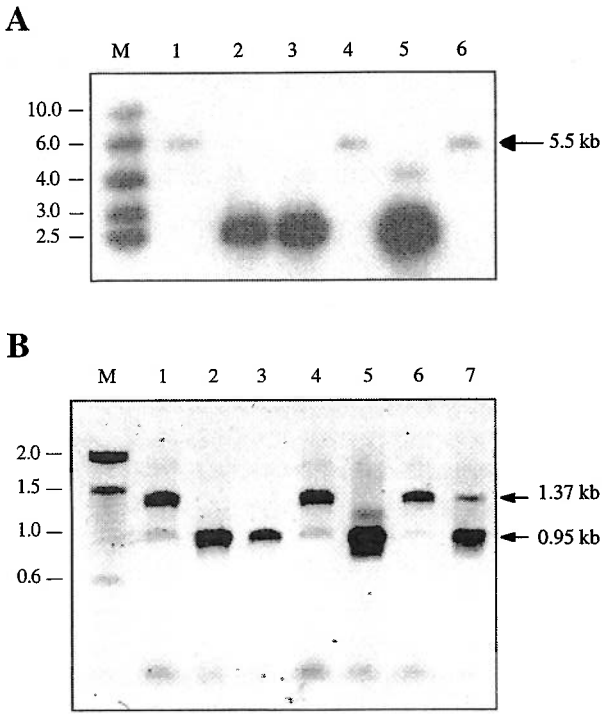

Figure 5: Control of *RIM15* deletion. **A.** Southern blot of yeast genomic DNA. The DNA was digested with *Xho*I and hybridized with a radiolabeled 0.52 kb fragment of *RIM15* as probe. The expected fragment size for the wild-type gene was 5.5 kb (see also Figure 3, Material and Methods). M: 1-kb DNA-marker (corresponding molecular weights are indicated to the left in kb); lane 1: AR1-1A (*RIM15*); lane 2: AR1-1B (*rim15Δ*); lane 3: AR1-1C (*rim15Δ*); lane 4: AR1-1D (*RIM15*); lane 5: AR2 (*rim15Δ/rim15Δ*); lane 6: YEF473 (*RIM15/RIM15*). **B.** PCR control. Genomic DNA was prepared and amplified by PCR as described in Material and Methods. The expected fragment size for wild-type *RIM15* was 1.37 kb, for *rim15Δ* 0.95 kb. M: 100 bp-ladder DNA-marker (corresponding molecular weights are indicated to the left in kb); lane 1: AR1-1A (*RIM15*); lane 2: AR1-1B (*rim15Δ*); lane 3: AR1-1C (*rim15Δ*); lane 4: AR1-1D (*RIM15*); lane 5: AR2 (*rim15Δ/rim15Δ*); lane 6: YEF473 (*RIM15/RIM15*); lane 7: AR1 (*RIM15/rim15Δ*).

was shown to segregate 2:2 as judged by the geneticin resistance of the resulting colonies. The size of wild-type and *rim15Δ* colonies did not differ significantly. Two geneticin-resistant segregants (AR1-1B, AR1-1C) were mated, resulting in a homozygous

diploid *rim15Δ* strain (AR2). Deletion of *RIM15* was confirmed in all strains by Southern blot analysis and/or PCR (Figure 5). These results indicate that *RIM15* is neither essential for germination nor for growth. As the heterozygous strain AR1 behaved like the wild type (see below) it could also be concluded that *RIM15* is recessive.

Effects of *RIM15* deletion

Growth of rim15Δ *cells on different carbon sources*

The effect of the deletion of *RIM15* upon the ability of cells to grow on different carbon sources was examined. In a first step all seven relevant strains, the diploid wild type YEF473, the two haploid wild-type segregants AR1-1A and AR1-1D, the heterozygous diploid strain AR1, the homozygous diploid *rim15Δ* strain AR2 and the two haploid *rim15Δ* segregants AR1-1B and AR1-1C were streaked out on solid SD media containing either glucose, fructose, galactose, glycerol, or ethanol as carbon source and incubated at either 27°C or 37°C. All strains grew well on glucose, fructose, galactose and ethanol at both temperatures. The only difference was the somewhat surprising observation that at 27°C the wild-type strains YEF473, AR1-1A and AR1-1D, and the heterozygous strain AR1 grew very slowly on glycerol while the *rim15Δ* strains grew better.

In a second experiment protein content and trehalose accumulation upon growth to stationary phase on media containing different carbon sources were determined (Table 5) for both the diploid wild-type strain YEF473 and the diploid *rim15Δ* strain AR2. The wild type accumulated most on glycerol (0.312 g trehalose /g protein) where hardly any growth was observed. It should be noted that these experiments were done on full YP media and not on SD media, therefore some of this growth can be attributed to the yeast extract used. Overall trehalose accumulation was higher on those carbon sources where little growth was observed. Deletion of *RIM15* resulted in low trehalose accumulation on all carbon sources tested. The exact factor of reduction in comparison with the wild type depended upon the carbon source used. When cells were grown on galactose they contained significantly more trehalose than on the other carbon sources tested (0.089 g/g protein on galactose, 0.034 g/g protein on glucose or fructose), making the 'low-trehalose-phenotype' of a *rim15Δ* strain somewhat less severe. This is important to note since galactose-containing medium was later used to induce expression of various constructs from plasmids containing a *GAL1* promoter (see Chapter III of this thesis). The *rim15Δ* strain accumulated between 60% (on galactose) to 87% (on maltose) less tre-

halose than the wild type. On average the reduction in trehalose accumulation in the mutant was about 80% compared with the wild type.

Table 5: *Protein content and trehalose accumulation on different carbon sources*

	RIM15/RIM15		*rim15Δ/rim15Δ*	
Carbon source	Protein (mg/ml)	Trehalose (g/g protein)	Protein (mg/ml)	Trehalose (g/g protein)
Glucose	1.129 ± 0.051	0.162 ± 0.014	1.237 ± 0.045	0.034 ± 0.019
Fructose	1.076 ± 0.048	0.189 ± 0.011	1.218 ± 0.015	0.034 ± 0.002
Sucrose	1.055 ± 0.021	0.131 ± 0.007	1.068 ± 0.013	0.024 ± 0.004
Raffinose	0.587 ± 0.013	0.284 ± 0.007	0.564 ± 0.004	0.046 ± 0.006
Maltose	0.238 ± 0.015	0.156 ± 0.012	0.250 ± 0.007	0.021 ± 0.012
Galactose	0.382 ± 0.003	0.222 ± 0.010	0.369 ± 0.006	0.089 ± 0.012
Galactose/ raffinose	1.184 ± 0.048	0.264 ± 0.025	1.196 ± 0.023	0.041 ± 0.005
Glycerol	0.399 ± 0.008	0.312 ± 0.020	0.696 ± 0.029	0.052 ± 0.003
Ethanol	0.776 ± 0.029	0.183 ± 0.021	0.622 ± 0.028	0.070 ± 0.003

Strains used were YEF473 (*RIM15/RIM15*) and AR2 (*rim15Δ/rim15Δ*). Cells were grown for 5 d on YP media containing the carbon source indicated in the table (1% [w/v] glucose, fructose, sucrose, raffinose, maltose, galactose; 1.5% [v/v] glycerol, ethanol; 0.5%/1% [w/v] galactose/raffinose). Protein content was measured as an indicator for the ability of the strains to grow on that particular carbon source. The results presented are the mean ± standard deviation of samples taken from three different cultures.

Cells deleted for RIM15 *are impaired in proper stationary phase entry*

The two-hybrid analysis initially suggested that Rim15 may interact with the Tre6P synthase (Tps1) and first experiments with the *rim15* disruption strain showed that a strain compromised for *RIM15* did not accumulate wild-type levels of trehalose in stationary phase. The *rim15Δ* strain AR2 was accordingly assayed for its ability to synthesize trehalose under different conditions. While it accumulated wild-type levels of trehalose during a 1-h heat shock at 42°C (0.345 ± 0.033 g/g protein vs. 0.377 ± 0.009 g/g protein in the wild type), it accumulated much lower amounts of trehalose in stationary

phase than the wild-type strain (Table 6). This defect in trehalose accumulation could be complemented by expressing the full length *RIM15* under its own promoter. As can be seen in Table 7, expression of YCplac33-*RIM15* (received from Guido Vogel) in AR2 led to normal accumulation of trehalose in stationary phase (0.238 g/g protein) when compared with the wild type containing the control plasmid (0.260 g/g protein). It can also be seen that the expression of an extra copy of *RIM15* in the wild-type background did not lead to an increase in the amount of trehalose accumulation (0.243 g/g protein compared with 0.260 g/g protein in the wild-type control). Since reintroduction of *RIM15* can complement the low-trehalose phenotype of the *rim15Δ* strain observed in stationary phase it can be concluded that the reduced trehalose accumulation in the *rim15Δ* strain is indeed due to the loss of *RIM15*.

Table 6: *Effects of* RIM15 *deletion*

	RIM15/RIM15[1]		*rim15Δ/rim15Δ*[1]	
	LOG	STAT	LOG	STAT
Enzymes and metabolites				
Tre6P synthase (μkat/g protein)	0.33	1.32	0.37	1.17
Trehalase (μkat/g protein)	0.09	0.17	0.05	0.16
Invertase (μkat/g protein)	2.00	12.93	1.93	12.06
SSA3-lacZ induction[2] (Miller units)	4.3	105.2	2.4	16.3
Trehalose (g/g protein)	<0.001	0.199	<0.001	0.009
Glycogen (mg/g protein)	1.63	38.53	0.93	11.36
Thermotolerance[3] (% survival)	0.13	35.6	0.07	0.03

[1] Wild-type and *rim15Δ/rim15Δ* strains were YEF473 and AR2, respectively. All experiments were carried out on YPD (2% [w/v] glucose for log, 1% [w/v] glucose for stationary cultures) medium using either log phase (LOG) or 4 d old stationary phase (STAT) cells, except where stated otherwise. Values represent means of at least three independent experiments; SDs were in each case < 10% of the corresponding means.

[2] β-Galactosidase activities were measured to monitor the induction of an *SSA3-lacZ* fusion gene (from plasmid pWB204Δ-236).

[3] Thermotolerance was measured as the percent survival following a heat shock for 8 min at 50°C (log phase cells) or 20 min (stationary phase cells) at 53°C.

Determining the *in vitro* activities of the two key enzymes of trehalose accumulation, Tre6P synthase and neutral trehalase however, revealed no significant difference between wild-type and *rim15Δ* strain, neither in stationary nor in log phase (Table 6). Considering that in stationary phase the *rim15Δ* strain contained considerably lower levels of trehalose than the wild type, it must be assumed that the *in vitro* activities of Tre6P synthase and/or trehalase at least for stationary phase cells do not correctly reflect the *in vivo* situation. Especially in the case of trehalase this seems very likely, since the activities determined *in vitro* are rather high in both strains, despite the fact that the wild-type contained high amounts of trehalose.

Table 7: *Expression of* RIM15 *complements the 'low-trehalose-phenotype' of a* rim15Δ *strain*

		Plasmids	
Strains	Relevant genotype	YCplac33	YCplac33-*RIM15*
YEF473	*RIM15/RIM15*	0.260 ± 0.007	0.243 ± 0.041
AR2	*rim15Δ/rim15Δ*	0.044 ± 0.009	0.238 ± 0.008

Strains were transformed with the single copy plasmid YCplac33, either containing a full length *RIM15* under its own promoter (YCplac33-*RIM15*) or no insert (YCplac33) (see also Material and Methods). Trehalose content (g/g protein) was determined in stationary phase after growth for 3 d on SD media containing 0.5% (w/v) glucose. The results shown are the mean ± standard deviation of three single transformants each.

Since the effects of *rim15Δ* seemed to manifest themselves only in stationary phase, a number of other phenotypic traits characteristic of stationary phase cells (for a review see Werner-Washburne *et al.*, 1993; Werner-Washburne *et al.*, 1996) were examined. Cells entering stationary phase accumulate trehalose and glycogen, acquire thermotolerance and induce a number of genes e.g. *SSA3*, *HSP12*, *HSP26*, *UBI4*, and *ADH2* (see also below). Depletion of glucose also leads to the transcriptional activation of a number of enzymes, such as the sucrose hydrolyzing enzyme invertase.

Glucose derepression occurred normally in a *rim15Δ* strain, the activity of invertase under glucose repressed (log-phase) and derepressed (stationary phase) conditions did not differ between the *rim15Δ* cells and the wild type (Table 6). However, it was found that loss of *RIM15* impaired cells in their ability to accumulate wild-type levels of glyco-

gen (reduced by 70%), to induce *SSA3* expression (reduced by 85%; measured by induction of an *SSA3-lacZ* fusion gene) and to acquire thermotolerance (reduced by 99%) (Table 6). Taken together, these results imply that Rim15 is required for the induction of a number of physiological adaptations characteristic of stationary cells and consequently indispensable for proper stationary phase entry.

Stationary rim15Δ *cells are capable of inducing trehalose synthesis upon heat shock*

One striking effect of a *RIM15* deletion was the lack of trehalose accumulation in stationary phase. Even though the Tre6P synthase activities determined *in vitro* were almost the same for wild-type (1.32 μkat/g protein) and *rim15Δ* strain (1.17 μkat/g protein), the latter did not contain wild-type levels of trehalose. The obvious question arising from this observation was whether the *rim15Δ* strain was generally unable to activate its Tre6P synthase in stationary phase *in vivo*. In order to resolve this question, it was attempted to determine whether the enzymes for trehalose synthesis could be activated by heat shock in stationary phase cells so that trehalose accumulation would be induced and if so,whether protein synthesis was necessary.

Stationary cells of YEF473 and AR2 were subjected to a series of different treatments followed by a heat shock at 42°C (Table 8). It is known that in wild-type cells a heat shock induces synthesis of trehalose in the presence of glucose in the medium. The treatments chosen were (i) heat shock, (ii) addition of glucose to a final concentration of 2% (w/v) followed by a heat shock, (iii) addition of glucose in the presence of the translation inhibitor cycloheximide (50 μg/ml) followed by a heat shock, or (vi) addition of cycloheximide followed by a heat shock. Without the addition of glucose heat shock did not cause an increase in trehalose levels in either wild-type or *rim15Δ* strain. However, when glucose was added to the cultures prior to heat shock, both strains were able to accumulate large amounts of trehalose (0.385 g/g protein in the wild type, 0.314 g/g protein in the *rim15Δ* strain). This accumulation was not dependent upon protein synthesis, since the presence of cycloheximide had no effect on the amount of trehalose synthesized during the heat shock (0.368 g/g protein in the wild type, 0.341 g/g protein in the *rim15Δ* strain).

These results can be interpreted in such a way that deletion of *RIM15* causes no general inability to synthesize trehalose *in vivo*. While the enzymes are present in the cells and trehalose synthesis is triggered by heat shock this trigger seems to be lacking when *rim15Δ* cells are grown to stationary phase. This makes it likely that the phenotypes observed in the *rim15Δ* strain including defects in trehalose synthesis, in the induction of *SSA3* expression, and in the acquisition of thermotolerance are due to an interruption of the signal transduction chain that induces cells to enter stationary phase.

Table 8: *Trehalose content in stationary phase cells of wild-type and* rim15Δ *strains subjected to a heat shock*

	RIM15[1]	*rim15Δ*[2]
Treatments		
Control[3]	0.143 ± 0.053	0.005 ± 0.006
Heat shock[4]	0.115 ± 0.015	0.010 ± 0.013
Glucose/ heat shock[5]	0.385 ± 0.037	0.314 ± 0.058
Glucose/ cycloheximide/ heat shock[6]	0.368 ± 0.050	0.341 ± 0.085
Cycloheximide/ heat shock[7]	0.124 ± 0.037	0.022 ± 0.035

[1] Strains used were AR1, AR1-1A and AR1-1D.
[2] Strains used were AR2, AR1-1B and AR1-1C.
[3] Cells grown to stationary phase (3 d) on YPD containing 1% (w/v) glucose. For the different treatments 3 ml aliquots were taken from these stationary cultures.
[4] Cells were subjected to heat shock at 42°C for 1 h.
[5] Glucose was added to a final concentration of 2% (w/v), and cells then subjected to heat shock for 1 h at 42°C.
[6] Glucose was added to a final concentration of 2% (w/v) together with 50 µg/ml cycloheximide, and cells then subjected to heat shock for 1 h at 42°C.
[7] Cycloheximide was added to a final concentration of 50 µg/ml, and cells then subjected to heat shock for 1 h at 42°C.

Effects of RIM15 *deletion during carbon or nitrogen starvation*

Because all the data accumulated up to this point indicated an important role of Rim15 for entry into stationary phase and most likely also in nutrient signaling, it seemed important to take a closer look at the response of the *rim15Δ/rim15Δ* (AR2) mutant to nutrient starvation in comparison with the wild type (YEF473). Two different conditions were chosen, one where carbon, the other where nitrogen was the limiting factor.

For the induction of carbon starvation, cells were grown on full medium containing 1% (w/v) glucose. Between 0 d to 20 d samples were taken at the times indicated in Figure 6. At each of the time points, trehalose content (Figure 6A), the percentage of budded cells (Figure 6B) and the survival rate (Figure 6C) were determined. As shown before, trehalose accumulation in the *rim15Δ* strain was low (0.013 g/g protein) when compared with the wild-type strain (0.134 g/g protein). The trehalose accumulation in the wild type

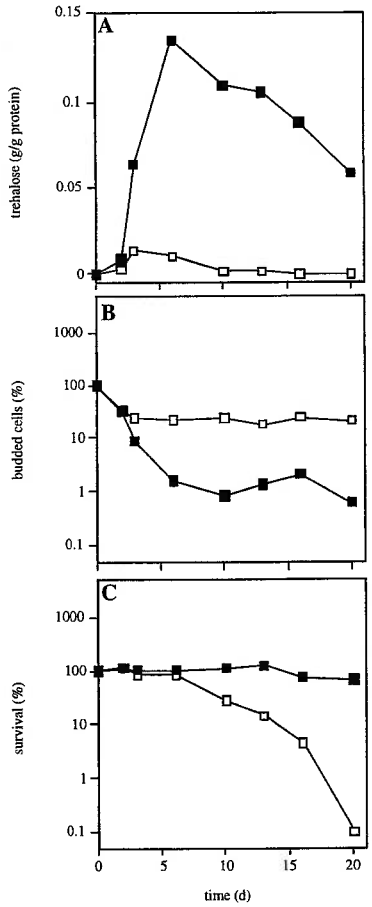

Figure 6: Effects induced by carbon starvation. Strains YEF473 (*RIM15/RIM15*; closed squares) and AR2 (*rim15Δ/rim15Δ*; open squares) were grown on full YP medium with 1% (w/v) glucose and samples were taken at the times indicated in the figure. Parameters determined were: **A.** accumulation of trehalose; **B.** percentage of budded cells; **C.** survival of cells.

peaked after 6 d and then started to decrease but never reached levels as low as the *rim15Δ* strain during the course of the experiment. Upon growth to stationary phase cells leave the cell cycle and enter a resting stage called G_0. At this stage, the cells of a culture are predominantly unbudded, so that the number of budded cells can be taken as an indicator for whether cells have entered G_0 or not. The percentage of budded cells after 2 d was the same in wild type and *rim15Δ* mutant (66%), but while this number decreased to

eventually 15% in the wild-type strain, it remained around 40% in the mutant. In addition to counting the number of budded cells, it was also recoreded whether budded cells were before, during or after mitosis. This classification was achieved by staining the DNA with propidium iodide and then counting budded cells with one nucleus as *before* nuclear division, with two nuclei as *after* nuclear division, and budded cells with one dividing nucleus as *during* nuclear division. From 6 d on more than 90% of the budded wild-type cells were in the stage after nuclear division, in the *rim15Δ* mutant however, 50% of the budded cells were still before or during nuclear division. This again suggests that deletion of *RIM15* negatively influences proper G_0 entry. For the graph in Figure 6B only those cells that were before or during mitosis were counted as true budded cells. This graph shows that the number of budded cells in the wild type decreased upon entry into stationary phase, reaching around 33% after 3 d and remained at about 1% for the rest of the experiment. The number of budded cells before or during nuclear division in the *rim15Δ* strain only decreased to about 25% during the first 6 d and then remained at this value until the end of the time course. Since the number of cells did not increase anymore during this period (data not shown) it can be concluded that cells carrying the *RIM15* deletion arrested randomly during the cell cycle instead of properly entering G_0 like the wild type. Sensitivity to carbon starvation was then determined by observing the percentage of survival of the two strains during the time course. The wild-type strain lost viability only to a very limited extent (66% viability after 20 d). The *rim15Δ* strain however, had lost more than 70% viability already after 10 d and this value continued to decrease continually until it was 0.1% after 20 d. This observation strongly supports the conclusions drawn from the determination of the number of budded cells that deletion of *RIM15* renders cells unable to properly enter stationary phase.

The results obtained from the assessment of cell cycle phase as described above are further supported by the results of a FACS analysis. FACS analysis or flow cytometry, by use of a specifically DNA-binding dye (propidium iodide), allows the determination of the DNA content and thereby of the cell cycle phase of individual cells. Typically the results are displayed in a diagram with the amount of DNA per cell on the x-axis and the number of cells for each amount of DNA on the y-axis. Cells in G_1 form a peak and cells in G_2 and M phase combine to form another peak at twice the value since the DNA has been replicated. Between these two peaks fall the cells with various amounts of DNA between G_1 and G_2, typical of the S phase. Cells in G_0 can not be separated from those in G_1 since they contain the same amount of DNA.

The same samples that were used to count the budded cells in the different phases of the cell cycle were also used for flow cytometry. As can be seen in Figure 7A, wild-type cells at 3 d are still about evenly distributed between G_1 and G_2 (postdiauxic phase), from 6 d on however, only one peak appears that corresponds to cells in G_1, meaning in this case that cells have probably entered G_0. Deletion of *RIM15* clearly affects this entry

into G_0 (Figure 7B). The FACS results show two peaks until the end of the time course, corresponding to cells in G_1 and G_2. The rather high background fluorescence in both strains is a problem often observed in stationary phase cells, especially if they were grown on YPD (Stephen Helliwell, personal communication). The results of the flow

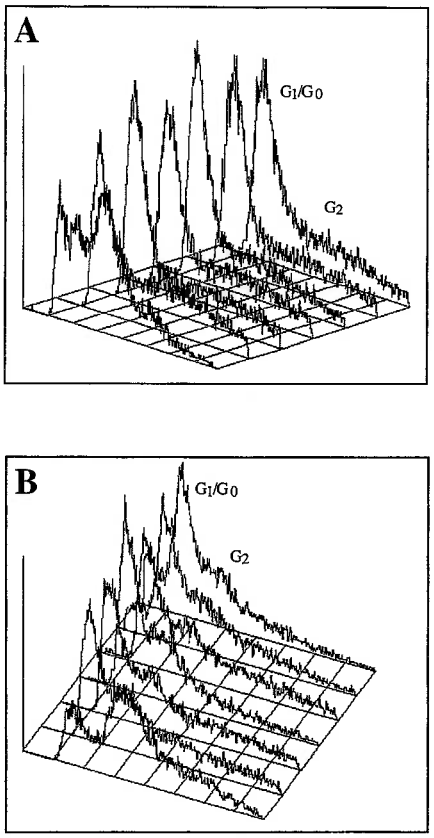

Figure 7: DNA content of cells growing to stationary phase on rich YP medium with 1% (w/v) glucose. The DNA content of diploid strains YEF473 (*RIM15/RIM15*) and AR2 (*rim15Δ/rim15Δ*) was determined by flow cytometry after propidium iodide staining as described in Material and Methods. Results are plotted as the relative number of events (number of cells) on the vertical axis against fluorescence (DNA content) on the horizontal axis. Cells in G_1/G_0 phase correspond to the left-hand, cells in G_2 to the right-hand peak. Graphs from front to back in both panels indicate samples taken after 2, 3, 6, 10, 13, 16, and 20 d of growth.

cytometry analysis together with those of the microscopic examination of the state of the nuclei before, during or after mitosis indicate that loss of *RIM15* disturbs proper entry into stationary phase and causes cells to arrest randomly at different points in the cell cycle.

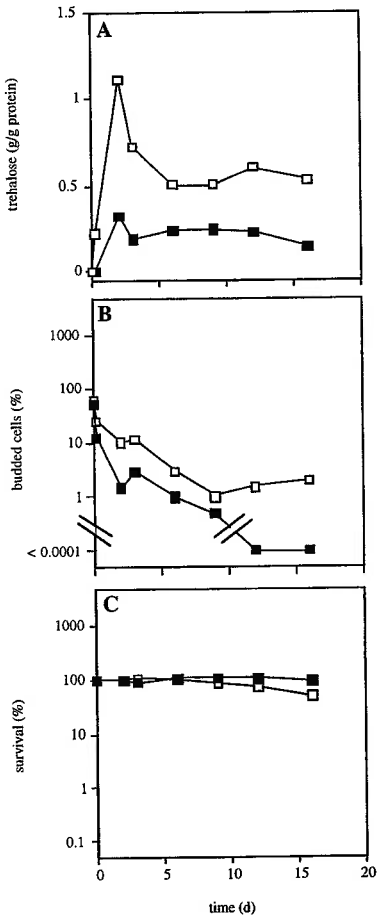

Figure 8: Reactions of strains YEF473 (*RIM15/RIM15*; closed squares) and AR2 (*rim15Δ/rim15Δ*; open squares) to nitrogen starvation. Cells were pregrown on SD medium with 2% (w/v) glucose and the appropriate amino acids until mid-log phase and then shifted to SD medium without nitrogen source and 4% (w/v) glucose to induce nitrogen starvation. Samples were taken at the times indicated in the figure. **A.** Trehalose accumulation; **B.** percentage of budded cells and **C.** survival of both strains were determined.

Nitrogen starvation was induced in YEF473 and AR2 by shifting cells pregrown to mid-log phase on SD medium containing 2% (w/v) of glucose to SD medium containing 4% (w/v) glucose and no nitrogen source. Again samples were taken during the course of the experiment (16 d) at the times indicated in Figure 8. Trehalose content (Figure 8A), number of budded cells (Figure 8B) and survival rate (Figure 8C) were determined. Trehalose accumulation was high in the wild type, reaching its peak after 2 d (1.109 g/g protein) and then decreasing to about 0.5 g/g protein at 9 d. The overall trehalose accumulation was much higher than the values measured during carbon starvation. Under nitrogen limitation the mutant contained less trehalose then the wild type but still accumulated substantial amounts of the disaccharide. The number of budded cells initially decreased similarly in both strains, but the *rim15Δ* strain retained about 1.5% of budded cells whereas this number in the wild type decreased to almost zero. Survival during nitrogen starvation was also affected in the *rim15Δ* strain. The wild type remained fully viable until 16 d, but the *rim15Δ* strain started to lose viability around 9 d, and after 16 d only 50% of the cells survived.

The effect of the *RIM15* deletion was differently severe depending upon the limiting factor forcing cells to enter stationary phase. Overall, nitrogen and carbon starvation caused a similar phenotype with respect to trehalose accumulation, number of budded cells, and loss of viability. However, this phenotype manifested itself less strongly if cells were grown under nitrogen limiting conditions.

Loss of RIM15 *abolishes derepression of genes normally induced during diauxic shift*

Diagnostic for the pleiotropic phenotype caused by the deletion of *RIM15* is the disability of the cells to respond to nutrient limitation by properly entering G_0. This can be interpreted in such a way that Rim15 acts as a component of a nutritional signal transduction pathway.

To further analyze this possibility, the transcription pattern of a number of genes including *SSA3*, *HSP12*, *HSP26*, *UBI4* and *ADH2* was studied in wild-type (YEF473) and *rim15Δ* (AR2) cells. Expression of these genes is known to become induced during or shortly after the diauxic shift (reviewed in Werner-Washburne *et al.*, 1989). Transcription levels were therefore compared in cells growing on rich, glucose based medium from exponential phase through diauxic shift, postdiauxic phase and eventually to stationary phase. In the wild-type strain the pattern of transcription was as expected (Figure 9). The *rim15Δ* strain however was shown to be seriously defective for the transcriptional derepression of *SSA3*, *HSP12*, and *HSP26*, but not for *ADH2* and *UBI4*. This once more implies that deletion of *RIM15* interrupts transmission of a signal that eventually leads to stationary phase adaptations.

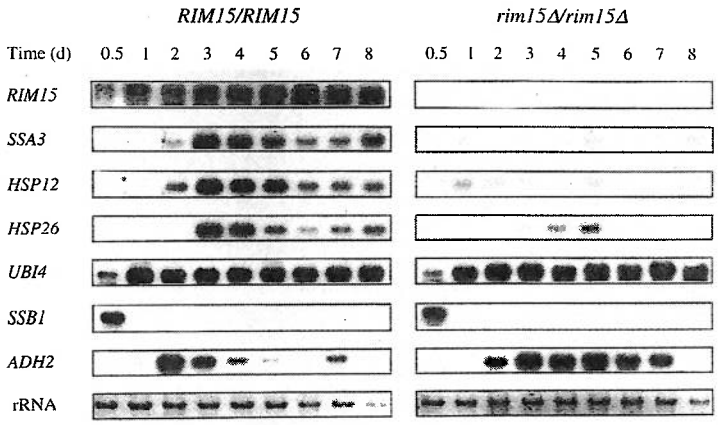

Figure 9: Northern blot analysis of gene expression in wild-type (YEF473) and *rim15Δ* (AR2) cells during growth to stationary phase. Total RNA prepared from cells in exponential phase (0.5 d), diauxic shift (1 d), postdiauxic phase (2-3 d), and stationary phase (4-8 d) was probed with *RIM15*, *SSA3*, *HSP12*, *HSP26*, *UBI4*, *SSB1* and *ADH2*. Equal loading (10 µg/lane) and integrity of RNA was verified by ethidium bromide staining (rRNA).

A closer look at the genes negatively affected in their expression levels by *rim15Δ* (*SSA3*, *HSP12*, and *HSP26*) reveals that all these genes are under negative control of cAPK (Kurtz *et al.*, 1986; Praekelt and Meacock, 1990; Boorstein and Craig, 1990b). The two genes that did not remain repressed in the *rim15Δ* strain, *UBI4* and *ADH2* are also under negative control of cAPK (Tanaka *et al.*, 1988; Cherry *et al.*, 1989), however, they differ from the other genes studied in some ways. For *UBI4* it has been shown that its expression is correlated with low cAMP levels but unlike *SSA3*, *HSP12*, *HSP26*, and *ADH2* it is already present during exponential growth and its transcription does not increase as strongly at the diauxic shift (Tanaka *et al.*, 1988). *ADH2* expression was even enhanced in a *rim15Δ* strain. In contrast to *SSA3*, *HSP12*, and *HSP26*, *ADH2* is known to be regulated by the transcription factor Adr1, which in turn is phosphorylated and thereby deactivated by cAPK (Cherry *et al.*, 1989). This and the fact that *SSA3* expression was normal in an *adr1* mutant make it likely that regulation of *SSA3* and *ADH2* occurs in a different branch of the Ras/cAMP pathway (Werner-Washburne *et al.*, 1989).

The cold-inducible *SSB1* is known to be strongly repressed upon entry into stationary phase (Werner-Washburne *et al.*, 1989). Both, wild-type and *rim15Δ* strain displayed normal repression of *SSB1* expression and no difference was detected between the two strains.

From the results of this experiment it can be deduced that Rim15 activity is required in cells entering stationary phase in order to achieve transcriptional derepression or activation of *SSA3*, *HSP12*, and *HSP26*, genes known to be under negative control of cAPK. It is therefore possible that Rim15 may consitute an element of the Ras/cAMP nutrient signaling pathway.

Deletion of RIM15 *results in sporulation deficiency*

The phenotype of *rim15Δ* suggests that Rim15 may be an element of a nutrient signaling pathway. It has been shown that without functional *RIM15* cells are unable to induce adaptations necessary for survival under starvation, consequently, they do not enter stationary phase properly. It is known that limitation of different nutrients affects yeast cells differently. The lack of a carbon source in the medium usually promotes stationary phase entry. However, diploid cells lacking both a fermentable carbon source and nitrogen, enter meiosis and produce spores. Accordingly, if Rim15 is an element of a general nutrient sensing pathway it could be expected that the deletion of *RIM15* in a diploid strain would also affect its sporulation frequency.

Table 9: *Sporulation frequency and trehalose content in sporulation cultures*

Strain	Relevant genotype	Sporulation (%)	Trehalose (g/g protein)
YEF473	*RIM15/RIM15*	31.1 ± 5.3	0.475 ± 0.041
AR2	*rim15Δ/rim15Δ*	0.6 ± 0.5	0.176 ± 0.032
AR1	*RIM15/rim15Δ*	25.8 ± 5.2	0.445 ± 0.038

Strains were grown on YPD for 2 d at room temperature and then shifted to sporulation media (1% K-acetate [w/v] plus 10 mg/ml of required amino acids). Asci were counted after 6 d. From each culture at least 300 cells were counted. The trehalose content was determined in the same cultures. The results shown are the mean ± standard deviation of six cultures per strain.

Sporulation was induced in the three diploid strains YEF473 (*RIM15/RIM15*), AR1 (*RIM15/rim15Δ*), and AR2 (*rim15Δ/rim15Δ*) by shifting them to sporulation medium and incubating them for 6 d. After this time at least 300 cells were counted under the microscope and the number of asci was determined (Table 9). At the same time the trehalose content in the sporulation cultures was analyzed (Table 9). Sporulation frequency in the

wild type was 31.1 ± 5.3% which was not significantly different from the sporulation frequency of the heterozygous strain AR1 (25.8 ± 5.2%). Interestingly, the homozygous *rim15Δ* mutant was defective for normal sporulation (0.6 ± 0.5%). The trehalose contents of sporulation cultures of YEF473 and AR1 were similar (0.475 ± 0.041 g/g protein and 0.44 ± 0.038 g/g protein), while those of AR2 contained significantly less trehalose (0.176 ± 0.038 g/g protein). These results were confirmed by Vidan and Mitchell (1997) who found a strong reduction of the ability to sporulate as one consequence of loss of *RIM15*. Thus, the *RIM15* gene is required for sporulation and may therefore be a transducer of a general starvation signal in *S. cerevisiae*.

Deletion of RIM15 *does not inhibit the transduction of a glucose signal*

Addition of glucose to derepressed yeast cells triggers a rapid transient increase in the cAMP-level. It was demonstrated that the Ras/cAMP pathway is involved in mediating this glucose-induced cAMP-signal (see Thevelein, 1991). As one of the consequences of this signal, neutral trehalase becomes activated via cAPK-dependent phosphorylation,

Table 10: *Induction of trehalase activity and trehalose hydrolysis in a wild-type and a* rim15Δ *strain by a glucose signal*

	Time (min)	*RIM15/RIM15*	*rim15Δ/rim15Δ*
Trehalase (μkat/g protein)	0	0.145 ± 0.035	0.146 ± 0.024
	30	0.355 ± 0.014	0.332 ± 0.018
Trehalase induction		2.170 ± 0.400	2.240 ± 0.280
Trehalose (g/g protein)	0	0.234 ± 0.058	0.012 ± 0.002
	30	0.191 ± 0.079	0.023 ± 0.014

Strains used were YEF473 (*RIM15/RIM15*) and AR2 (*rim15Δ/rim15Δ*). Cells were grown at 27°C to stationary phase (4 d) on YPD with 1% (w/v) glucose. At 0 min half of each culture was harvested and resuspended in the same volume of fresh YPD with 2% (w/v) glucose and incubated at 27°C (glucose signal), the other half was kept as a control at 27°C. At 0 min and after 30 min samples were taken and assayed for trehalase activity and trehalose content. The induction of trehalase activity after the glucose signal was calculated. The experiment was repeated three times, mean ± standard deviation of these repeats are shown.

and trehalose is degraded. The ability of *rim15Δ* cells to activate neutral trehalase after the addition of glucose to a derepressed culture could therefore indicate whether loss of *RIM15* compromises signal transduction via the Ras/cAMP pathway.

Glucose was added to derepressed cultures of YEF473 (*RIM15*) and AR2 (*rim15Δ*), and the activity of neutral trehalase and the trehalose content was assayed at the start of the experiment and after 30 min, controls were left without addition of glucose. During the course of the experiment trehalase activity was equally induced in response to the glucose added in both strains (Table 10). The activation of trehalase *in vivo* was weakly reflected by hydrolysis of trehalose in the wild-type strain, where the trehalose content decreased from 0.234 g/g protein to 0.191 g/g protein. The already low trehalose level of the *rim15Δ* strain remained unchanged. No changes in trehalase activity or trehalose content were observed in the controls (data not shown). Earlier experiments using the disruption mutant yielded the same results (data not shown). The fact that the activation of trehalase in response to a cAMP signal is independent of *RIM15* suggests that if Rim15 has a function in the Ras/cAMP pathway it does not act upstream of cAPK.

DISCUSSION

The *RIM15* gene was identified in a two-hybrid screen as encoding an interactor of Tps1, the Tre6P synthase. Primary sequence analysis revealed that *RIM15* codes for a new serine/threonine protein kinase with a catalytic domain that closely resembles the cAPK catalytic domain of yeast (encoded by *TPK1*, *TPK2*, and *TPK3*). Unlike the cAPK catalytic subunit, Rim15 has large amino- and carboxy-terminal regions that show no homology to other *S. cerevisiae* proteins in the data base. These domains might be of a regulatory function as it has been speculated for the Tpk-homolog Sch9 (Toda *et al.*, 1988). In favor of this idea it was found that these regions as well as the large insert between kinase subdomains VII and VIII contain five consensus sites for phosphorylation by cAPK (one of these sites is part of a consensus site for protein kinase C dependent phosphorylation). Protein kinases in general are known to be important regulatory proteins and key components of many signaling pathways in eukaryotic cells. They activate or inactivate their substrates through the phosphorylation of serine, threonine or tyrosine residues. Since one of the aims of this study was to isolate potential regulators of trehalose synthesis and since regulation through phosphorylation-dephosphorylation mechanisms had been proposed for this enzyme (see Introduction), it seemed highly interesting to study this newly identified protein kinase gene in more detail and to assess whether it could indeed play a role in trehalose synthesis. Therefore it was decided to initiate the analysis of the two-hybrid interactors with the *RIM15* gene.

The significance of the other identified interactors with Tps1 or Tps2 remains to be investigated. Apart from the known subunit of the Tre6P synthase/phosphatase complex, Tsl1 (Vuorio *et al.*, 1993), none of the interactors has been described in detail in the literature. Both Tps1-interactors YPL032C and YBR061C show significant homologies to known genes, YPL032C to the *S. cerevisiae PAM1* gene and YBR061C to the *E. coli ftsJ* gene. The meaning of these homologies remains unclear since the function of *PAM1* as well as of *ftsJ* has not been clarified. The last class of interactors, YHR202W, interacting with Tps2, has no significant homology to any known gene in the EMBL data base. It might be an interesting candidate to study since it also strongly interacted with other subunits of the Tre6P synthase/phosphatase complex, namely Tps1 and Tps3 and therefore might be an effector of the whole enzyme complex.

In an attempt to estimate whether the degree of interaction determined by the two-hybrid system parallels the degree of interaction detected by biochemical techniques, it was found that these two, *in vivo* and *in vitro* measurements, were generally correlated with one another (Estojak *et al.*, 1995). For the results of our study this means that one could with caution assume that the two-hybrid interaction of Tps1 with Rim15 represents

a rather low, of Tps1 with YPL032C and YBR061C an intermediate and of Tps2 with Tsl1 and YHR202W a high affinity interaction.

Despite the fascinating identification of the *RIM15* gene as potential regulator of trehalose synthesis, a number of open questions in view of the original aim of the study remain. Only one of the known subunits of the Tre6P synthase/phosphatase complex was isolated from the library, even though they were shown to interact in a directed two-hybrid analysis (Reinders *et al.*, 1997). This could be a general problem of the two-hybrid assay, possibly resulting from the potentially altered tertiary structure of the proteins, from low protein stability or low expression levels of any of the constructs (Fields and Sternglanz, 1994) or could have its origin in the structure of the library used. The same reasons may explain the failure to identify any proteins involved in the glycolytic pathway, even though the pleiotropic phenotype resulting from *tps1Δ* indicates that Tps1 has in addition to its role in trehalose synthesis also a part in glycolysis (Thevelein and Hohmann, 1995). Another explanation could also be that a direct interaction of Tps1 with elements of the glycolytic pathway is not necessary for it to exert its presumed regulatory task. Two of the proposed models for Tps1-dependent control of glycolytic flux, namely the phosphate-recovery model and the hexokinase-inhibition model (see also Introduction, Chapter I) assume regulation rather through activity of the enzyme than by physical interaction with other proteins. As a general cautionary note it must also be kept in mind that failing to detect an interaction between two given proteins in a two-hybrid analysis does not necessarily reflect their lack of interaction under physiological circumstances (Fields and Sternglanz, 1994). To avoid some of the pitfalls of a two-hybrid library screen, future studies to address the proposed role of Tps1 as part of the glycolytic machinery should make use of directed two-hybrid analyses as it has been done for the subunits of the Tre6P synthase/phosphatase complex (Reinders *et al.*, 1997).

The fact that *RIM15* was identified as an interactor of Tps1 and that it was found to encode a protein kinase, made it very tempting to speculate that Tps1 could be a substrate for Rim15-dependent phosphorylation. As described in the Introduction, regulation of trehalose synthesis by phosphorylation/dephosphorylation of the Tre6P synthase had been discussed but so far no conclusive evidence to support this theory had been provided (Panek *et al.*, 1987; Vandercammen *et al.*, 1989). One of the first steps to address the speculation that Tps1 could be regulated by phosphorylation through Rim15 was the study of the consequences of loss of Rim15 activity for the synthesis of trehalose. It was found that cells without *RIM15* only contained low levels of trehalose in stationary phase. This result indirectly supports the idea of Rim15-dependent Tps1 regulation. The somewhat contradictory result that the *in vitro* activity of Tre6P synthase was found to be similar in wild-type and *rim15Δ* cells, despite their difference in trehalose accumulation, might be explained by modifications of the Tre6P synthase during the sampling procedure (*e.g.* proteolysis; Vicente-Soler *et al.*, 1991; Vuorio *et al.*, 1993). Due to the complex structure

of the Tre6P synthase/phosphatase complex it has to be expected that the *in vitro* determination of Tre6P synthase activity not always reflects the *in vivo* situation. This problem has already been encountered and discussed before (Reinders *et al.*, 1997; see also Discussion in Chapter I of this thesis).

Remarkably, Rim15 was dispensable for trehalose accumulation in response to a mild heat shock, even in stationary phase cells. Hence, it can be concluded (i) that *rim15Δ* cells are not essentially incapable of synthesizing trehalose and (ii) that regulation of trehalose synthesis during stationary phase entry and during heat shock is mediated by different mechanisms. As to the first conclusion drawn, it has been discussed above that the trehalose synthesizing enzymes are present in the *rim15Δ* cells and that they are active *in vitro*. Therefore, loss of *RIM15* does not prevent the synthesis of, nor does it result in irreversibly inactivated Tre6P synthase. In fact, it can even be inferred that Rim15 is not essential for trehalose synthesis *per se*. However, Rim15 seems to be essential for the transmission of the signal triggering trehalose synthesis in cells entering stationary phase. This speculation leads to the second conclusion drawn above and thereby back to the debate about the regulatory mechanisms of trehalose synthesis. In the Introduction different models suggested for the control of trehalose synthesis have been described. It had also been implied that the mechanisms allowing trehalose accumulation in response to a mild heat shock and upon stationary phase entry might not be identical. The results of this study present further evidence for this assumption. It has clearly been shown that stress-induced trehalose accumulation is regulated through a pathway independent of Rim15. Even more, it can be deduced that regulation in stationary phase must contain a posttranslational element, since in the case of the *rim15Δ* strain, the synthesizing enzymes were present (as seen by their activities *in vitro*), however they were not active *in vivo*. Considering that the evidence for the interaction of Rim15 and Tps1 remains to be indirect at present, it must be taken into account that the low trehalose level in stationary *rim15Δ* cells could also be explained by an increased activity of trehalase instead of decreased activity of Tre6P synthase *in vivo*. This objection is supported by the observation that trehalase activities determined *in vitro* from stationary cells are rather high even in wild-type cells, contrary to the fact that these contain high amounts of trehalose. It seems reasonable to assume that *in vitro* and *in vivo* activities of trehalase in this case are not identical. The exact explanation for the observations therefore remains to be discovered.

The general picture emerging from the physiological studies presented in this chapter, makes it likely that Rim15 is part of a nutrient signaling pathway. Upon nutrient exhaustion, Rim15 is required for the induction of a number of physiological adaptations associated with proper stationary phase entry. Consequently, loss of *RIM15* leads to a pleiotropic phenotype if cells are grown under carbon limiting conditions, including the failure to accumulate trehalose and glycogen, to derepress or activate expression of *SSA3*, *HSP12*, and *HSP26*, to arrest in G_1 and to acquire heat and starvation resistance.

Moreover, diploid *rim15Δ/rim15Δ* cells were impaired for sporulation. Most interestingly, this phenotype of a *rim15Δ* strain is, overall, reminiscent of phenotypes of mutations in the Ras/cAMP pathway that cause unregulated activation of cAPK, such as *bcy1* and $RAS2^{val19}$. Bcy1 is the regulatory subunit of the *S. cerevisiae* cAPK (Matsumoto *et al.*, 1982; Toda *et al.*, 1987a). Cells lacking a functional *BCY1* gene display unbridled cAPK activity that is totally unresponsive to cAMP. A similar phenotype is the consequence of $RAS2^{val19}$, a dominant active allele of *RAS2* that leads to elevated cAMP levels through enhanced adenylate cyclase activity and thereby to increased activity of cAPK (Kataoka *et al.*, 1984; Toda *et al.*, 1985). Both these mutations cause low trehalose and glycogen levels, low thermotolerance and a rapid loss of viability in stationary phase. In diploid strains they also cause low sporulation efficiency which has prompted the suggestion that the Ras/cAMP pathway and the pathway leading to the initiation of meiosis could be linked (Matsuoka *et al.*, 1983). All the phenotypical features described above were also determined for *rim15Δ* strains. Moreover, deletion of *RIM15* brought about a defect in the induction of certain genes, namely *SSA3*, *HSP12*, and *HSP26* which have been shown to be under the negative control of cAPK. Taken together these results invite to speculate that Rim15 might actually be involved in nutrient signaling through the RAS/cAMP pathway.

It should be noted that *rim15Δ* cells also had some phenotypes distinct from cells with an unregulated cAPK. Deletion of *RIM15* neither resulted in a particular susceptibility to nitrogen starvation nor in impaired gluconeogenesis (as determined by the ability to grow on gluconeogenic carbon sources and to induce *ADH2* and invertase), both of which are usually associated with high cAPK activity.

To sum up sequence analysis and extensive studies of the phenotype of cells without functional Rim15, have provided evidence that Rim15 is part of a nutrient signaling pathway, influencing the cell's decision to enter stationary phase. A number of highly interesting questions arise in this context:

(i) Is Rim15 an element of the Ras/cAMP pathway and if so, at which point of the pathway does it act ?

(ii) Does Rim15 have protein kinase activity and what are its substrates ?

(iii) How is Rim15 activity regulated ?

(iv) What is the relationship between Rim15 and Tps1, and does Rim15 regulate trehalose accumulation through the modulation of Tps1 activity ?

Answers to these questions will undoubtedly prove useful in providing a detailed picture of the functions of Rim15 in both trehalose synthesis and nutrient signaling. Therefore, Chapter III is dedicated to experiments which aim to elucidate these questions.

CHAPTER III

SACCHAROMYCES CEREVISIAE cAMP-DEPENDENT PROTEIN KINASE CONTROLS ENTRY INTO STATIONARY PHASE THROUGH THE Rim15 PROTEIN KINASE

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Andres Wiemken and Claudio De Virgilio

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INTRODUCTION

Metabolism, proliferation, and development of microorganisms, are controlled by the presence or absence of nutrients in the surrounding medium. In *S. cerevisiae*, one of the most important extracellular signals is glucose. Addition of glucose to derepressed cells triggers a variety of regulatory phenomena which involve several different signaling pathways (for a review on nutrient signaling see Thevelein, 1994). Besides the main glucose repression pathway, the Ras/cAMP pathway is the most intensively studied nutritional signaling pathway. The Ras/cAMP pathway is important for regulation of growth, cell cycle progression, and development in response to nutritional signals, via its central enzyme cAPK. Mutations affecting the activity of cAPK result in a pleiotropic phenotype, indicating the existence of many potential substrates for this kinase. Cells deficient for cAPK activity arrest in G_1, accumulate enhanced levels of glycogen and trehalose, constitutively express a set of genes normally induced in stationary phase (e.g. *SSA3, HSP12, HSP26, CTT1, UBI4*, and *ADH2*), show enhanced stress resistance, and undergo meiosis in rich medium (provided they are diploids). On the other hand, cells with an overactive cAPK fail to arrest in G_1 upon nutrient limitation, contain low levels of glycogen and trehalose, remain sensitive to heat shock and nutrient starvation, are unable to grow on non-fermentable carbon sources, and fail to sporulate. Based on these observations it was suggested that the Ras/cAMP pathway is involved in regulating the transition between mitotic growth and a distinct quiescent state, analogous to the G_0 state described for mammalian cells. Accordingly, low cAPK activity would trigger entry into G_0, whereas high cAPK activity would prevent access to G_0, making cAPK a kind of universal integrator of nutrient availability in *S. cerevisiae* (for reviews see Broach and Deschenes, 1990; Thevelein, 1994).

The signal transduction pathway regulating the activity of cAPK in budding yeast has been studied in detail. Initially, interest has been fueled by the discovery that this pathway contains small G-proteins which are encoded by two genes, *RAS1* and *RAS2* that are homologs of mammalian *ras* genes. *ras* genes, which are highly conserved throughout all eukaryotic species, have an important role in the etiology of mammalian cancer. In fact, they are a main type of oncogene found in naturally occurring and artificially induced mammalian tumors. Consequently, it was hoped that by defining the function of *RAS* genes in yeast, a better understanding of mammalian *ras* genes and their oncogenic alleles would be achieved. However, the initial euphoria has diminished since it has been demonstrated that in *S. cerevisiae* Ras proteins function predominantly as modulators of adenylate cyclase while metazoan Ras proteins, and interestingly also the

Ras proteins of *S. pombe,* appear to exert their function independently of cAMP (Toda *et al.*, 1985; Fukui *et al.*, 1986; Gibbs and Marshall, 1989).

The *S. cerevisiae* Ras proteins are active in their GTP-bound state and inactive in their GDP-bound state. The activity of Ras is modulated by the products of the *CDC25* and the *IRA1* and *IRA2* genes. Cdc25, the GTP-exchange factor (GEF), is a positive regulator of Ras activity. It stimulates the intrinsic GTPase-activity of Ras, thereby promoting the removal of GDP bound to Ras and its replacement with free GTP. Ira1 and Ira2 (Ira) are structural and functional homologs of mammalian GTPase-activating protein (GAP). They stimulate Ras GTPase-activity and thereby downmodulate Ras activity. A dominant active allele of *RAS2*, $RAS2^{val19}$ is, like its mammalian oncogenic counterpart, impaired in its GTPase activity and insensitive to regulation by Ira (Kataoka *et al.*, 1984). In its active state the Ras proteins of *S. cerevisiae* are capable of stimulating adenylate cyclase (encoded by *CDC35/CYR1*), the enzyme responsible for the synthesis of cAMP. Degradation of cAMP is achieved by the low- and high-affinity phosphodiesterases (encoded by *PDE1* and *PDE2*, respectively). The synthesis of cAMP leads to the activation of cAPK. This protein kinase is a heterotetramer, consisting of two regulatory (encoded by *BCY1*) and two catalytic subunits (encoded by the homologous genes *TPK1*, *TPK2*, and *TPK3*). In the absence of cAMP, Bcy1 is tightly bound to the catalytic subunits, restraining their activity. When Bcy1 binds cAMP, it dissociates from the catalytic subunits, thereby relieving the inhibition, the enzyme is then in its active state. In cells growing on medium containing a fermentable carbon source such as glucose, cAMP-levels are high and consequently cAPK is active (Ras function and the Ras/cAMP pathway have been reviewed in Tatchell, 1986; Gibbs and Marshall, 1989; Broach and Deschenes, 1990; Broach, 1991; Thevelein, 1992).

Despite the fact that the components of the Ras/cAMP pathway have been identified and well characterized, neither the exact mechanism of signal perception at the cell membrane nor most of the precise targets affected by the pathway are known. However, it has been established that the primary triggers of the Ras/cAMP pathway are rapidly-fermentable sugars and intracellular acidification. In response to these triggers the level of cAMP in the cells increases, which leads to the activation of cAPK (Mbonyi *et al.*, 1990; Van Aelst *et al.*, 1990; Thevelein, 1991). This observation is consistent with the idea that the Ras/cAMP pathway is responsible for signaling the nutritional status of the cell. Downstream targets of cAPK probably include enzymes involved in the metabolism of storage carbohydrates, in glycolysis and gluconeogenesis, in phospholipid metabolism, and in cAMP synthesis, as well as transcription factors. But so far only very few of these targets of cAPK have been unequivocally identified. One example for a well established substrate of cAPK, as has been mentioned before, is the trehalose-hydrolyzing enzyme trehalase. This enzyme has been shown to be phosphorylated and thereby rapidly activated by cAPK *in vitro* (see Introduction Chapter 2).

The question whether the Ras/cAMP pathway may influence not only mobilization but also synthesis of trehalose in a direct manner has been raised repeatedly. In favor of this assumption, several studies have demonstrated that mutations affecting the activity of the Ras/cAMP pathway also affect the accumulation of trehalose. (Tenan *et al.*, 1985; Toda *et al.*, 1985; Hottiger *et al.*, 1989). More importantly, it has also been shown that strains with increased cAPK activity had decreased Tre6P synthase activity and *vice versa* (Panek *et al.*, 1987; François *et al.*, 1991). However, the precise mechanism behind these results remains unclear, and so far it has not been convincingly shown that cAPK directly regulates Tre6P synthase activity (Vandercammen *et al.*, 1989). With the identification of Rim15 as a two-hybrid interactor of Tps1 and as a putative element of the Ras/cAMP pathway, as discussed in the previous chapter, it is tempting to speculate that Rim15 could actually provide a link between trehalose synthesis and the Ras/cAMP pathway.

In this chapter, attempts to corroborate this speculation by detection of direct physical interaction between Rim15 and Tps1 are presented. Moreover, this chapter contains genetic and biochemical evidence for a model in which Rim15 functions directly downstream and under negative regulation of cAPK to control entry into stationary phase. Experiments described in this chapter have been performed in collaboration with Claudio De Virgilio and with the technical assistance of Niels Bürckert.

RESULTS

Analyses of epistasis with the Ras/cAMP pathway

The results described and discussed in the previous chapter had led to the suggestion that Rim15 could have a role in the Ras/cAMP pathway. This suggestion was mainly supported by the finding that loss of *RIM15* leads to a number of phenotypes that are remarkably similar to those encountered in cells with unregulated cAPK, for instance *bcy1* or *RAS2^{val19}*. In order to corroborate this suggestion, analyses of epistasis were performed, investigating the consequences of overexpression of *RIM15* on the one hand and loss of *RIM15* on the other hand in a number of strains carrying mutations affecting the Ras/cAMP pathway.

Deletion of RIM15 *rescues the thermosensitive growth defect of a* cdc35-10 *strain and suppresses phenotypes caused by total loss of cAPK*

In order to understand better the relationship of Rim15 to the Ras/cAMP pathway, it was determined whether loss of *RIM15* could suppress a defect in the adenylate cyclase. This was done by deleting the *RIM15* gene in a *cdc35-10* background (strain PD6517). The *cdc35-10* mutation is a temperature sensitive allele of adenylate cyclase. Cells carrying this mutation grow at 27°C but are unable to do so at 35°C. Three colonies deleted for *RIM15* in this background along with three colonies of PD6517 were tested for growth in liquid YPD medium at the non-permissive temperature. As expected, *cdc35-10* cells failed to grow at 35°C (OD600 after 2 d = 0.15 ± 0.06). Loss of Rim15, however, enabled the cells to grow at 35°C (OD600 after 2 d = 4.47 ± 0.26). Accordingly, it can be concluded that Rim15 may act in the Ras/cAMP pathway downstream of adenylate cyclase.

To further clarify the position of Rim15 in the Ras/cAMP pathway, it was examined whether loss of *RIM15* could alleviate the lethality of total loss of cAPK, conferred by the disruption of all three genes coding for the catalytic subunit of protein kinase A (*TPK1*, *TPK2*, *TPK3*). *RIM15* was deleted in the heterozygous diploid strain S7-7A x S7-5A (*TPK1/tpk1 TPK2/tpk2 TPK3/tpk3* [*TPK/tpk*]). The resulting strain (NB13) was allowed to sporulate and tetrad analysis was performed. Genotypes of viable spores were scored according to the auxotrophic requirements, and those of nonviable spores were predicted from those of the other spores in the same tetrad, assuming normal 2:2 segregation. As expected, no viable progeny carrying disruptions in all *TPK* genes could be re-

covered. However, spores germinated and grew if they also carried *rim15Δ* in addition to *tpk1 tpk2 tpk3*. Therefore, deletion of *RIM15* suppresses the complete loss of cAPK. This result was further confirmed by a second experiment. A *tpk1 tpk2 tpk3 rim15Δ* (*tpk rim15Δ*) strain was transformed with either a plasmid allowing galactose-inducible expression of *RIM15* (YCpIF2-*RIM15*) or with a control plasmid (YCpIF2) and tested for growth on either glucose- or galactose-containing medium. As expected, cells containing YCpIF2-*RIM15* grew readily on glucose but failed to grow on galactose, while the cells containing the control plasmid had no such growth defect (Figure 1). Again this confirms that *rim15Δ* suppresses loss of cAPK activity.

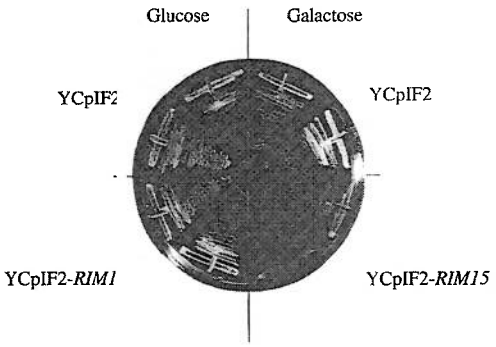

Figure 1: Deletion of *RIM15* suppresses the lethality of complete loss of cAPK. *RIM15* was placed under control of a galactose-inducible promoter on plasmid YCpIF2-*RIM15* and reintroduced into strain NB13-14D (*tpk1 tpk2 tpk3 rim15Δ*). Two clones were tested for their ability to grow on glucose- or galactose-containing SD medium. YCpIF2 designates the empty control vector.

Having shown that *rim15Δ* suppresses the growth defect of *tpk* strains, in a next step it was tested whether a *tpk rim15Δ* strain would also be impaired for other stationary phase adaptations, that is whether it behaved like a *rim15Δ* mutant. The expression of genes induced upon diauxic shift, namely *SSA3*, *HSP12*, *HSP26*, *UBI4*, and *ADH2*, was studied in a wild-type (*TPK RIM15*), a *rim15Δ* (*TPK rim15Δ*) and a *tpk rim15Δ* strain in log and in stationary phase (2 d and 4 d after glucose depletion, Figure 2). This set of genes has previously been shown to be under negative control of cAPK (Kurtz *et al.*, 1986; Tanaka *et al.*, 1988; Cherry *et al.*, 1989; Werner-Washburne *et al.*, 1989; Praekelt and Meacock, 1990). In the wild type *SSA3*, *HSP12*, and *HSP26* were repressed in log cells but derepressed in stationary phase. Deletion of *RIM15* resulted in re-

pression of these genes both in log and stationary phase, irrespective of the presence or absence of the *TPK* genes. The expression of *UBI4*, *ADH2*, and of *SSB1*, a marker gene for exponential phase, was not affected by loss of either *RIM15* or *TPK*, largely confirming earlier results on gene expression in a *rim15Δ* strain (Chapter II, Figure 9).

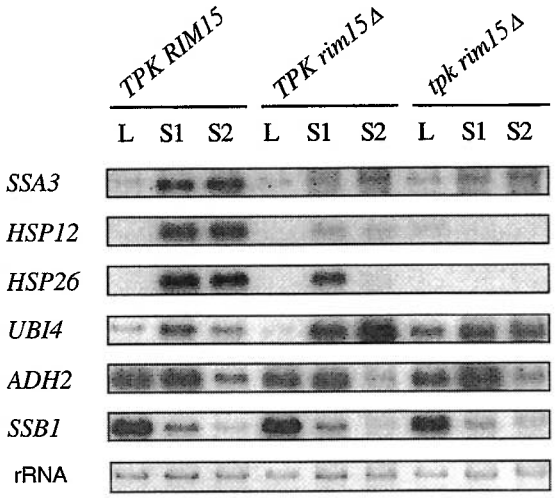

Figure 2: Analysis of gene expression in exponentially growing and stationary phase *TPK RIM15* (SP1), *TPK rim15Δ* (NB13-1D), and *tpk rim15Δ* (NB13-14D) cells. Cells were grown on YPD medium containing 1% (w/v) glucose as carbon source and total RNA was prepared from cells in exponential (L) and in stationary phase (S1: 2 d after glucose exhaustion; S2: 4 d after glucose exhaustion). The RNA was separated on agarose gels, blotted to nitrocellulose and probed with *SSA3*, *HSP12*, *HSP26*, *UBI4*, *ADH2*, and *SSB1*. Equal loading (10 µg) and RNA integrity were verified by ethidium bromide staining (rRNA).

Other phenotypes associated with stationary phase and with low cAPK activity include accumulation of trehalose and glycogen, high thermotolerance, and increased tolerance of prolonged starvation. These characteristics were therefore analyzed and compared in wild-type (Table 1, row 1), *rim15Δ* (Table 1, row 2), and *tpk rim15Δ* (Table 1, row 5) cells. Strains deleted for *RIM15* were always defective for the accumulation of trehalose in stationary phase, regardless of the presence or absence of *TPK*. Strains bearing *tpk rim15Δ* also displayed low thermotolerance, like it is known from *rim15Δ* strains (Table 1, rows 2, 5). However, not all phenotypes associated with loss of cAPK activity

Table 1: *Effects of* rim15Δ *and* yak1 *in* TPK, tpk, and tpk^{w} *strain backgrounds*

Row	Relevant genotype	Trehalose (g/g protein)	Glycogen	Thermotolerance (%)	Survival (%)	Growth (h^{-1})
1	*TPK RIM15 YAK1*	0.181 ± 0.077	+	26.5 ± 6.4	90.3 ± 15.7	0.278 ± 0.016
2	*TPK rim15 YAK1*	< 0.001	-	0.1 ± 0.1	4.6 ± 5.4	0.265 ± 0.006
3	*TPK RIM15 yak1*	0.056 ± 0.024	+	4.5 ± 2.0	61.2 ± 13.4	0.278 ± 0.016
4	*TPK rim15 yak1*	<0.001	-	0.1 ± 0.1	5.5 ± 2.9	0.274 ± 0.006
5	*tpk rim15 YAK1*	0.059 ± 0.052	+++	6.3 ± 1.6	50.7 ± 9.8	0.162 ± 0.026
6	*tpk RIM15 yak1*	0.347 ± 0.020	+++	51.4 ± 22.6	87.9 ± 18.1	0.169 ± 0.029
7	*tpk rim15 yak1*	0.032 ± 0.028	+++	6.7 ± 2.5	55.8 ± 11.1	0.109 ± 0.006
8	*tpk^{w} RIM15 YAK1*	0.917 ± 0.032	+++	90.5 ± 22.5	114.0 ± 17.5	n.d.
9	*tpk^{w} rim15 YAK1*	0.371 ± 0.009	+++	108.8 ± 21.2	81.1 ± 18.6	n.d.

Trehalose content and thermotolerance were measured in strains grown on full liquid medium (YPD) for 4 d to stationary phase. Thermotolerance was determined as survival following a heat shock for 20 min at 50 °C. Glycogen content was measured qualitatively by iodine staining of cells grown on YPD agar for 3 d. Survival was determined from cultures grown for 10 d on full liquid medium. The growth rate was measured during exponential growth on glucose. All experiments were repeated three times with one (*TPK RIM15 yak1*: CDV80-1D; *tpk^{w}*: RS13-58A-1), two (*TPK rim15 YAK1*: CDV80-2B and CDV80-7B; *TPK rim15 yak1*: CDV80-15D and CDV80-33B; *tpk RIM15 yak1*: CDV80-2D and CDV80-5A; *tpk rim15 YAK1*: CDV80-15A and NB13-14D; *tpk rim15 yak1*: CDV80-4A and CDV80-8A), or three (*TPK RIM15 YAK1*: CDV80-2C, CDV80-4B, and CDV80-4C; *tpk^{w} rim15 YAK1*: CDV81-3A, CDV81-11B, and CDV81-16D) independent strains with the same relevant genotype.

were suppressed by *rim15Δ*. Glycogen levels remained high in *tpk rim15Δ* cells, as verified by iodine staining. Also, *tpk rim15Δ* strains had a lower growth rate than *rim15Δ* strains (0.162 h^{-1} compared with 0.265 h^{-1} for the *rim15Δ* strain; Table 1, rows 2, 5), and they were more starvation resistant in stationary phase (50.7% survival after 10 d compared with 4.6% for the *rim15Δ* strain; Table 1, rows 2, 5). Summarizing, these results point to a model in which Rim15 acts downstream of cAPK, being responsible for a subset of physiological adaptations induced upon entry into stationary phase, including trehalose accumulation, acquisition of thermotolerance, and the induction of certain genes (*SSA3*, *HSP12*, and *HSP26*). Conversely, other stationary phase phenotypes, such as glycogen accumulation seem not to be induced through the activity of Rim15, suggesting control mechanisms which act through Tpk but without involving Rim15.

Effects of rim15Δ *in* TPK, tpk, *and* $tpk1^w$ *strains*

Interpretation of the described studies with the *tpk rim15Δ* mutant was rendered difficult by the fact that the phenotype of this strain can not be compared directly with the phenotype of a *tpk* strain, since the latter strain is not viable. This problem can be partially overcome with a special type of *TPK* mutant. Cells deleted for two of the three *TPK* genes and carrying a so-called 'wimpy' allele for the third, are viable and display attenuated cAPK activity. If, in addition, *BCY1* is disrupted, this low cAPK activity is also unregulated and cAMP-independent (Cameron *et al.*, 1988). Such a strain is considered to be the closest equivalent possible to a *tpk* strain. It seemed therefore interesting to analyze the effects of *rim15Δ* in such a *$tpk1^w$ tpk2 tpk3 bcy1* (*tpk^w*) background. Like with the *tpk rim15Δ* strain, stationary phase characteristics, such as the induction of *SSA3*, *HSP12*, *HSP26*, *UBI4*, and *ADH2* expression, along with the accumulation of trehalose and glycogen, and the induction of thermo- and starvation tolerance were investigated. Surprisingly, the effects of *rim15Δ* were not identical in a *tpk* and a *tpk^w* background.

As shown in Figure 3, the wild-type strain displayed normal repression/derepression in logarithmic and stationary phase of *SSA3, HSP12, HSP26, UBI4, ADH2*, and *SSB1*. Log-phase *tpk^w RIM15* cells were slightly derepressed for *SSA3, HSP12*, and *ADH2* expression but normal for *HSP26* and *SSB1*. Deletion of *RIM15* had no effect in *tpk^w* log-phase cells, except that *HSP26* was slightly derepressed in addition to *SSA3, HSP12*, and *ADH2*. Therefore, in log-phase *rim15Δ* did not show a detectable phenotype in both backgrounds.

Based on the finding that *tpk^w* strains responded properly to nutrient limitation, it has been suggested previously that cAMP-independent mechanisms may exist for controlling the starvation response (Cameron *et al.*, 1988). In accordance, transcription levels

of *SSA3, HSP12, HSP26, UBI4*, and *ADH2* were found to increase normally in *tpk*^{w} *RIM15* cells in stationary phase, while *SSB1* became repressed (Figure 3). In the *tpk*^{w} *rim15Δ* strain expression of *SSA3, HSP12*, and *HSP26* was diminished but not completely absent in stationary phase. This result indicates that the induction/derepression of these particular genes depends at least partially upon *RIM15*. Even more interesting, this is an unexpected difference to the findings with the *tpk rim15Δ* strain described above (Figure 2). The *tpk rim15Δ* strain showed no expression of *SSA3, HSP12*, and *HSP26*. at all in stationary phase. Obviously, *rim15Δ* suppresses *tpk*^{w} not to the same extent as *tpk* with respect to the expression of these genes.

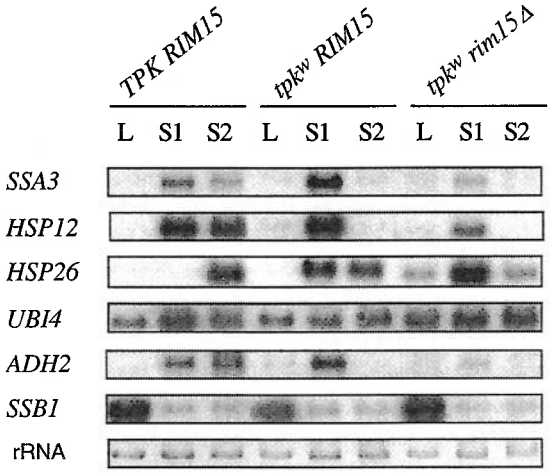

Figure 3: Analysis of gene expression in exponentially growing and stationary phase *TPK RIM15* (CDV80-4C), *tpk*^{w} *RIM15* (RS13-58A-1), and *tpk*^{w} *rim15Δ* (CDV81-16D). Cells were grown on YPD medium containing 1% (w/v) glucose as carbon source and total RNA was prepared from cells in exponential (L) and in stationary phase (S1: 2 d after glucose exhaustion; S2: 4 d after glucose exhaustion). The RNA was separated on agarose gels, blotted to nitrocellulose and probed with *SSA3, HSP12, HSP26, UBI4, ADH2*, and *SSB1*. Equal loading (10 µg) and RNA integrity were verified by ethidium bromide staining (rRNA).

Other stationary phase adaptations (trehalose and glycogen accumulation, starvation and thermotolerance) were also investigated in *tpk*^{w} and *tpk*^{w} *rim15Δ* cells (Table 1, rows 8, 9). The *tpk*^{w} strain hyperaccumulated glycogen and trehalose and was highly resistant to heat stress and prolonged (10 d) starvation. Deletion of *RIM15* in this background had no effect on the hyperaccumulation of glycogen and did not decrease starvation and heat

resistance of the cells. The trehalose content was lower in a *tpk*^{w} *rim15Δ* cells compared with *tpk*^{w} cells, but cells still contained rather high levels of this disaccharide when compared with the wild type (row 1) or *TPK rim15Δ* cells (row 2). The findings made with a *tpk rim15Δ* strain (see above) had already suggested that glycogen accumulation is not under control of Rim15, while the other phenotypic traits analyzed appeared to be strongly dependent upon Rim15. The findings with the *tpk*^{w} *rim15Δ* strain seem to contradict this idea because *rim15Δ* does not or only partially suppress the phenotype conferred by *tpk*^{w} with respect to trehalose accumulation, and acquisition of heat and starvation resistance. This seeming contradiction to the model placing Rim15 downstream and under negative control of cAPK may be reconciled if it is assumed that the unregulated 'wimpy' allele is also active during a phase when cAPK would normally be inactive (stationary phase) and that it may then interfere with targets normally not directly controlled by cAPK.

Overexpression of RIM15 *partially induces a starvation response in exponentially growing wild-type cells, complements* bcy1-1, *and exacerbates the growth defects at elevated temperatures of* cdc35-10 *cells*

As shown in *Chapter II*, deletion of *RIM15* renders cells unable to enter stationary phase properly. Therefore it was assumed that the activity of Rim15 is necessary for the signal transduction chain that controls the adaptations observed upon nutrient depletion from the growth medium. Consequently, overexpression of *RIM15* in exponentially growing wild type cells should induce a starvation response even in the presence of nutrients. This hypothesis was tested by using wild-type strain YEF473 bearing either a plasmid allowing galactose-inducible expression of *RIM15* under the *GAL1* promoter (YCpIF2-*RIM15*) or an empty control plasmid (YCpIF2). Both strains were assayed in logarithmic growth and stationary phase for their trehalose accumulation, *SSA3*-lacZ induction and thermotolerance (Table 2). Cells overexpressing *RIM15* showed a tenfold induction of trehalose, a threefold higher *SSA3* expression and a fivefold increase in thermotolerance when compared with the cells containing the empty control plasmid. Moreover, stationary phase cells overexpressing *RIM15* contained 50% more trehalose, showed 37% stronger *SSA3*-lacZ induction, and acquired higher thermotolerance levels when compared with the control cells (Table 2). These results confirm that the activity of Rim15 is an important positive regulator of the starvation response in yeast cells.

The results described in *Chapter II* had also demonstrated that deletion of *RIM15* results in a phenotype similar to the phenotype caused by unregulated constitutive activation of cAPK. Also, analyses of epistasis performed so far are consistent with Rim15

Table 2: *Effects of* RIM15 *overexpression*

		Trehalose (g/g protein)	*SSA3-lacZ* induction (Miller units)	Thermotolerance (% survival)
YCpIF2	LOG	0.006 ± 0.002	12.6 ± 3.0	0.9 ± 0.5
YCpIF2	STAT	0.238 ± 0.030	429.0 ± 61.9	77.1 ± 8.5
YCpIF2-*RIM15*	LOG	0.063 ± 0.009	37.0 ± 3.7	4.9 ± 1.6
YCpIF2*RIM15*	STAT	0.364 ± 0.027	589.0 ± 43.7	98.5 ± 18.0

Cells of strain YEF473 (wild type) containing either plasmid YCpIF2 (control) or YCpIF2-*RIM15* (overexpression of *RIM15*) were grown to log (LOG) or stationary phase (STAT; 4 d) on SD media containing 2% galactose and 1% raffinose to induce *GAL1*-driven transcription of the *RIM15* gene in YCpIF2-*RIM15*. Expression of *SSA3* was determined as β-Galactosidase activities resulting from the induction of an *SSA3-lacZ* fusion gene (from plasmid pWB204Δ-236). Thermotolerance was measured as survival following a heat shock for 4 min (log phase cells) or 20 min (stationary phase cells) at 50°C. Values repesent means ± SD of three independent experiments.

Table 3. *Suppression of* bcy1-1 *phenotypes by overexpression of* RIM15

		Trehalose (g/g protein)	*SSA3-lacZ* induction (Miller units)	Thermotolerance (% survival)
BCY1	YCp*ADH1*	0.39 ± 0.05	137.8 ± 37.5	39.8 ± 3.7
BCY1	YCp*ADH1-RIM15*	0.56 ± 0.11	299.9 ± 76.0	73.0 ± 2.4
bcy1-1	YCp*ADH1*	0.12 ± 0.06	1.4 ± 11.1	12.6 ± 2.9
bcy1-1	YCp*ADH1-RIM15*	0.24 ± 0.02	123.9 ± 25.7	43.0 ± 10.9

Strains SP1 (*BCY1*) and T16-11A (*bcy1-1*) containing either plasmid YCp*ADH1* (control) or YCp*ADH1-RIM15* (*RIM15*-overexpression) were grown to stationary phase (5 d) on SD-media containing 1% glucose. 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 20 min at 50°C. Values represent means ± SD of three to six independent experiments.

being under direct or indirect negative control of cAPK. Overexpression of *RIM15* should therefore revert the phenotypic effects associated with unbridled cAPK activity, as for instance in a *bcy1-1* strain. In order to test this assumption, strains T16-11A (*bcy1-1*) and SP1 (wild type) were transformed with either a plasmid allowing constitutive overexpression of *RIM15* under the *ADH1* promoter (YCp*ADH1-RIM15*) or a control plasmid (YCp*ADH1*). Cells were grown to stationary phase and trehalose content, *SSA3*-lacZ induction and thermotolerance were monitored (Table 3). Overexpression of *RIM15* in the *bcy1-1* strain was able to partially overcome the lack of trehalose accumulation (0.24 ± 0.02 g/g protein compared with 0.12 ± 0.06 g/g protein in the *bcy1-1* strain and 0.39 ± 0.05 g/g in the wild-type control), and to completely suppress the *SSA3*-expression defect (123.9 ± 25.7 compared with 1.4 ± 11.1 in the *bcy1-1* strain and 137.8 ± 37.5 in the wild-type carrying the control plasmid) and acquisition of thermotolerance (43.0 ± 10.9% survival compared with 12.6 ± 2.9% in the *bcy1-1* strain and 39.8 ± 3.7% in the wild-type carrying the control plasmid). Overexpression of *RIM15* in the wild-type background (*BCY1*) again led to overall higher trehalose accumulation, *SSA3* induction and survival after heat shock. Therefore, Rim15 is epistatic to Bcy1, indicating that it acts downstream of this protein in the Ras/cAMP pathway.

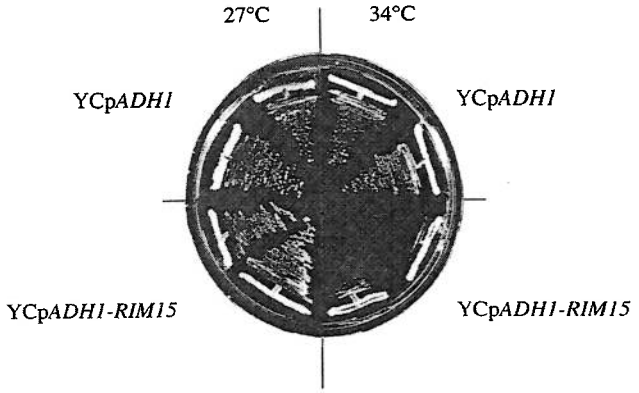

Figure 4: Overexpression of *RIM15* exacerbates the thermosensitive growth phenotype of a *cdc35-10* strain. Two clones of strain PD6517 (*cdc35-10*) bearing either the *RIM15*-overexpressing plasmid YDp*ADH1-RIM15* or the control plasmid YCp*ADH1* were incubated on SD medium at 27°C or 34°C for 3 d to monitor growth.

The effects of *RIM15* overexpression in *RAS2^{val19}* mutants which also have unbridled cAPK activity were ambiguous. While overexpression of *RIM15* (YCp*ADH1-*

RIM15) in the *RAS2^{val19}* strain JC482 partially suppressed the thermotolerance defect (44.9 ± 4.8% survival after 20 min at 50°C for cells containing YCp*ADH1-RIM15* and 15.0 ± 0.1% survival for cells expressing the control plasmid YCp*ADH1*) it only partially suppressed its defect in trehalose accumulation (0.084 ± 0.004 g/g protein compared to 0.060 ± 0.008 g/g protein in control cells). In contrast, overexpression of *RIM15* in the *RAS2^{val19}* strain TK161-R2V did neither suppress its thermotolerance nor its trehalose accumulation defect.

The same strain carrying a thermosensitive allele of adenylate cyclase (*cdc35-10*) that was used to test the effect of *rim15Δ* was also used to monitor the effect of *RIM15* overexpression. While cells carrying the control plasmid were able to grow both at 27°C and at 34°C, those that overexpressed *RIM15* grew at 27°C but failed to grow at 34°C. Therefore, overexpression of *RIM15* exacerbates the temperature sensitivity of a strain carrying the *cdc35-10* mutation (Figure 4). These results signify that the point of action of Rim15 lies downstream of the gene product of *CDC35*, which is consistent with all other experimental results obtained so far.

Epistatic analyses of RIM15 *and* YAK1*: Yak1 and Rim15 are not in the same pathway*

The *YAK1* gene had been identified in a screen for suppressors of the thermosensitivity of *ras* mutants and it was found to encode a protein kinase. Moreover, analogous to the deletion of *RIM15*, deletion of *YAK1* overcame the lethal growth defect of a *tpk* strain (Garrett and Broach, 1989). Current knowledge, however, places Yak1 in a pathway parallel to cAPK. One of the main arguments for this model is the fact that Yak1 activity is not inhibited by phosphorylation through cAPK *in vitro* (Garrett *et al.*, 1991). The phenotype of *tpk1 tpk2 tpk3* (*tpk*) *yak1* strains described in the literature resembles in some ways the one of *tpk rim15Δ* strains. Cells of both strains are able to grow, at a somewhat lower growth rate, and they accumulate high amounts of glycogen. It seemed reasonable to test the effects of simultaneous loss of Rim15 and Yak1. Strains carrying *rim15Δ* contained less trehalose and glycogen (Table 1, lanes 2, 5), were less thermotolerant and more starvation sensitive than strains carrying *yak1*, irrespective of the presence or absence of functional cAPK (Table 1, lanes 3, 6). Only the growth rates of *rim15Δ* and *yak1* strains were similar and loss of either gene conferred slow growth in a *tpk* background (growth rates were 0.162 for *tpk rim15Δ* and 0.169 for *tpk yak1*). The double mutants, *rim15Δ yak1* and *tpk rim15Δ yak1* (Table 1, lanes 4, 7), resembled *rim15Δ* cells with respect to trehalose content, thermotolerance, and starvation resistance. Therefore, judged by these phenotypic traits, the two mutations had no additive effect. The only exception was the growth rate of the double mutant without cAPK, which was decreased compared with the *tpk* strains carrying the single mutations of *RIM15* and *YAK1*,

respectively. These findings would be consistent with Rim15 being downstream of Yak1, either in a pathway parallel to the Ras/cAMP pathway or, taking in account our current knowledge on Rim15, in such a way that Rim15 could integrate signals from both cAPK and Yak1. Any interpretation must be made with caution, though, and would certainly require more data on the nature of the relationship between Yak1, Rim15, and cAPK.

Co-precipitation studies of Rim15 and Tps1 or Tpk1

Attempts to determine biochemical interaction between Rim15 and both Tps1 and Tpk1

Two-hybrid analysis had indicated that Rim15 and Tps1 could physically interact which was further corroborated by the finding that *rim15Δ* mutants were defective for stationary phase-induced trehalose synthesis. In parallel, analyses of epistasis indicated that Rim15 itself is a downstream effector of cAPK and may therefore also be physically associated with Tpk1. In order to further substantiate these findings, it was attempted to co-precipitate Rim15 with either Tps1 or Tpk1 in biochemical experiments. During the course of these experiments several obstacles were encountered, some of which could be tackled, others proved more difficult to overcome. The different attempts will be shortly described in the following paragraphs.

Since currently no antibodies are available against Rim15, this protein was expressed in *S. cerevisiae* as a fusion protein with glutathione S-transferase (GST). This 'tag' allows purification of the protein by affinity binding to glutathione sepharose. Likewise, Tps1 and Tpk1 were expressed as maltose-binding protein (MBP) fusions in *E. coli* which could be purified by their affinity to amylose. Based on the values from the two-hybrid interaction between Rim15 and Tps1 it was expected that the proteins only interact weakly with each other (Estojak *et al.*, 1995). Two-hybrid data on the strength of the interaction between Rim15 and Tpk1 are not available, since both DBD-fusions (DBD-Rim15 and DBD-Tpk1) caused very high background activities (results not shown).

Initially, attempts were made to purify full-length GST-Rim15 from cells grown for 2 d on SD medium with 2% (w/v) galactose and 1% (w/v) raffinose. However, while the cells containing the control plasmid expressed GST (readily detectable both on a coomassie stained SDS-gel [Figure 5, lane 1, left panel] and on immunoblots probed with anti-GST antibodies [Figure 5, lane 1, right panel]), no GST-Rim15 protein could be detected in extracts of cells expressing *GST-RIM15* (Figure 5, lane 2). Different conditions for the purification were tested but no apparent flaws in the purification protocol could be found which could explain this result. Since not even proteolytic fragments of GST-Rim15 could be detected, it was concluded that proteolysis during preparation of the ex-

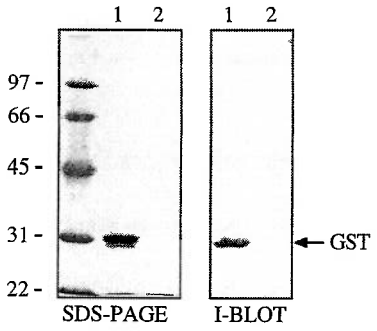

Figure 5: Full-length GST-Rim15 was not detectable in growing cells. Wild-type strain MH272-1D was transformed with YCpIF2-*GST* (GST) or with YCpIF2-*GST-RIM15* (GST-Rim15) and grown for 2 d on SD medium containing galactose and raffinose. Glutathione sepharose-affinity purified proteins from cells containing the GST control plasmid (lane 1) or the GST-Rim15 overexpressing plasmid (lane 2) were separated by SDS-PAGE (left panel) and analyzed by immunoblot (I-blot, right panel) using anti-GST antibodies. Protein sizes (indicated to the left in kDa) were determined by means of a protein standard.

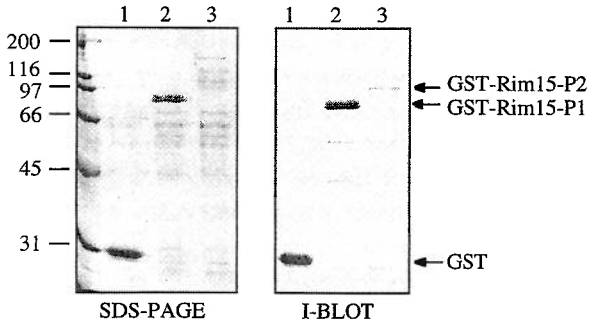

Figure 6: Immunoblot of truncated versions of GST-Rim15. Wild-type strain MH272-1D containing YCpIF2-*GST*, YCpIF2-*GST-RIM15-P1* or YCpIF2-*GST-RIM15-P2* was grown for 2 d on SD medium containing galactose and raffinose. Proteins were purified by affinity binding to glutathione sepharose and analyzed by SDS-PAGE (left panel) and immunoblot (I-blot, right panel). Lane 1: GST, lane 2: GST-Rim15-P1, lane 3: GST-Rim15-P2. Standard protein sizes are indicated to the left (in kDa).

tracts was not the main problem and that the GST-Rim15 protein rather may be subjected to high proteolytic degradation *in vivo*. In a next step it was then tested whether partial *GST-RIM15* fusions were translated into more stable protein products. Two constructs were made and tested, GST-Rim15-P1 (amino acids 761-1266) and GST-Rim15-P2 (amino acids 761-1770). Both constructs were detectable on coomassie-stained SDS-gels and immunoblots (Figure 6). However, the crucial step that eventually also made it possible to purify full-length GST-Rim15, proved to be an extension of the culture time to more than 2 d. In stationary phase cells GST-Rim15 seemed to be more stable, still some proteolysis was always detected (see Figure 7A).

Once expression and purification of full-length GST-Rim15 had been successful, the next step was to achieve efficient electro-transfer of this large protein to nitrocellulose. Buffer conditions, blotting time, and voltage were optimized, in order to transfer as much as possible of GST-Rim15 without losing any of the smaller sized proteins. Still, full-length GST-Rim15 transferred not very efficiently, while shorter proteolytic fragments could be blotted easily (as judged by staining the SDS-gels with coomassie blue after the transfer). Therefore, on immunoblots the full-length protein was always slightly under-represented.

In a next step it was then possible to assay for coprecipitation of GST-Rim15 with MBP-Tps1 or MBP-Tpk1. Affinity purified GST-Rim15 or GST alone as a control, were combined with either MBP-Tps1, MBP-Tpk1, MBP-Tim12 (an unrelated nuclear protein), or MBP (control). Interactions were always determined in both directions, *i.e.* precipitation of each mix was performed with both glutathione sepharose and amylose resin. Provided that two proteins interact with each other, both types of precipitation should yield the same result; for example MBP-Tps1 should co-precipitate with the glutathione sepharose-bound GST-Rim15, and GST-Rim15 should co-precipitate with the amylose resin-bound MBP-Tps1. Detection of the fusion proteins was achieved by immunoblot, using either anti-GST or anti-MBP antibodies.

The results of one experiment are displayed in Figure 7. Full length GST-Rim15 as well as proteolytic fragments and GST protein were purified efficiently with glutathione sepharose (Figure 7A). As can be seen, both MBP-Tps1 and MBP-Tpk1 co-precipitated with GST-Rim15 (Figure 7C, lanes 5 and 6). However, they also co-precipitated with the GST control (Figure 7C, lanes 1 and 2). Moreover, the unrelated controls MBP-Tim12 and MBP protein both also bound to GST-Rim15 and GST (Figure 7C, lanes 3, 4, 7, and 8). Interestingly, MBP-Tpk1 only bound very weakly to the GST control. Still, since the unrelated controls both co-precipitated with the target protein, no statement is possible whether the interactions observed between MBP-Tpk1/MBP-Tps1 and GST-Rim15 reflect true *in vivo* interactions. Pull-down with amylose resin only yielded the MBP-tagged proteins alone (Figure 7B), no co-precipitating GST-fusion pro

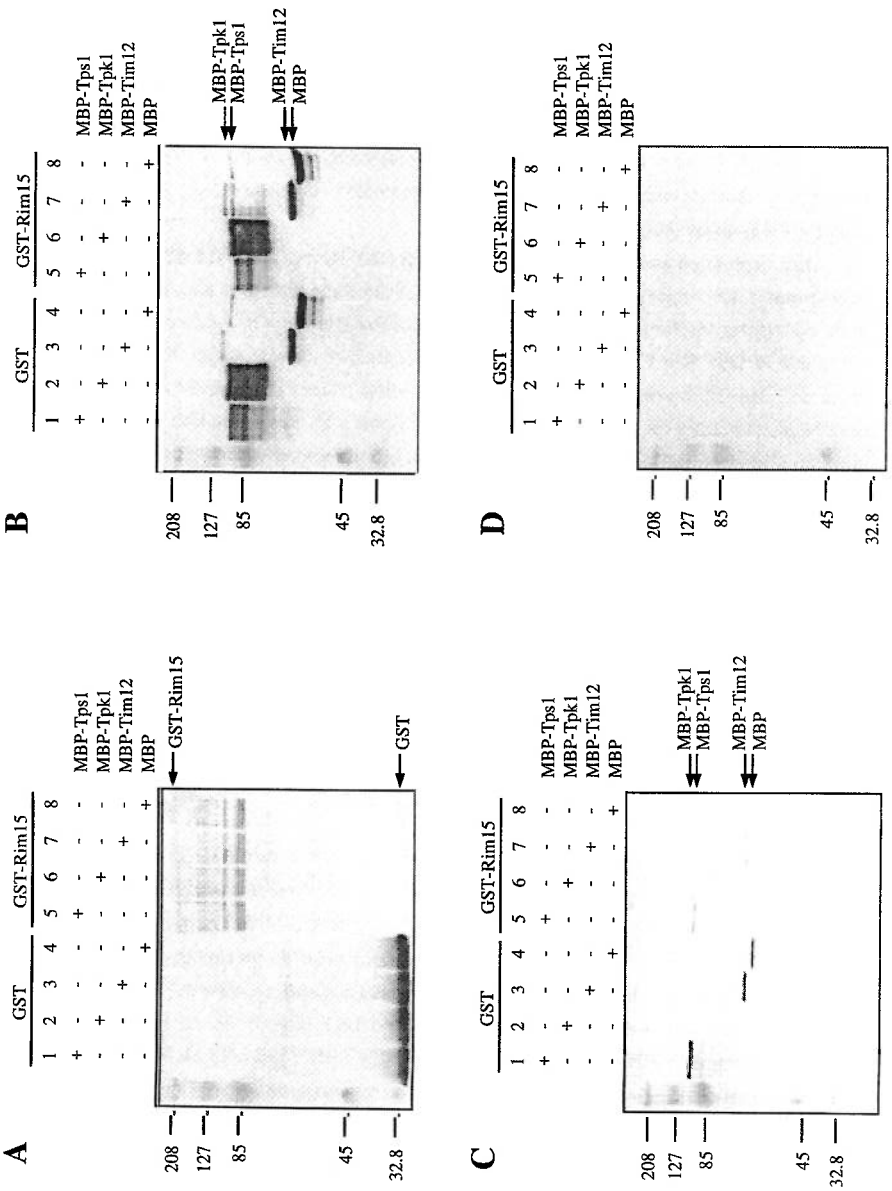
A
B
C
D
GST
GST-Rim15
1 2 3 4 5 6 7 8
MBP-Tps1
MBP-Tpk1
MBP-Tim12
MBP
GST-Rim15
GST
MBP-Tpk1
MBP-Tps1
MBP-Tim12
MBP
208
127
85
45
32.8

Figure 7 (see last page): Co-precipitation analysis of GST-Rim15 and MBP-Tps1 or MBP-Tpk1. Glutathione sepharose affinity purified GST-Rim15, or GST as control, were incubated with either a potential interactor (MBP-Tps1 or MBP-Tpk1) or with an unrelated control (MBP or MBP-Tim12) and proteins were precipitated with either glutathione sepharose (A, C) or with amylose resin (B, D). Samples were analyzed by immunoblot using anti-GST (A, D) or anti-MBP (B, C) antibodies. Protein sizes (in kDa) are indicated to the left of each panel.

tein could be detected (Figure 7D). Apparently, under the conditions chosen for the exper-
iment, MBP was able to bind to the glutathione-sepharose matrix (it did probably not bind to the GST-tag since in that case one would expect to also detect co-precipitation in the corresponding amylose resin pull-down experiment, Figure 7D). Therefore, these results neither confirm nor exclude that an *in vivo* interactions exists between Rim15 and Tps1 or Tpk1. Future attempts should employ different tags to circumvent the observed non-specific binding of MBP to glutathione sepharose. One basic problem may also be the fact that both Tps1 and Tpk1 were expressed in *E. coli* instead of in yeast. This could result in modifications or changes in protein folding that do not allow to detect interactions as they would occur with the endogenous proteins. Co-expression of *GST-RIM15* together with *TPS1* and *TPK1*, respectively, may circumvent this obstacle. Another strategy might be the integration of a tagged Rim15 into the genome and expression under its own promoter. Both Rim15-Tps1 and Rim15-Tpk1 co-precipitation could then be studied under native conditions, using if possible, antibodies specific for these proteins to detect them on immunoblots. This would also allow to check whether the proposed interactions exists only during a certain growth phase, *e.g.* only during the diauxic shift.

Kinase activity of Rim15

Rim15 has protein kinase activity that can be inhibited by in vitro *phosphorylation through cAPK*

RIM15 specifies a polypeptide that includes regions conserved among serine/threonine protein kinases. Thus, there seemed no doubt that Rim15 functions as a protein kinase. In order to actually detect such protein phosphorylating activity *in vitro*, Rim15 was expressed as a GST-fusion protein in yeast. As described above, the GST-tag allowed precipitation of GST-Rim15 from crude extracts by means of affinity binding to glutathione sepharose. In addition, commercially available anti-GST antibodies allowed detection of the tagged protein on immunoblots.

To determine whether Rim15 may function as a protein kinase, autophosphorylation activities of wild-type and of a mutant Rim15 derivative were compared. The mutant derivative ($Rim15^{K823Y}$) carries a substitution replacing the invariant lysine in kinase subdomain II with tyrosine. This lysine residue lies in the ATP-binding site, and its substitution impairs both ATP and peptide substrate binding (Gibbs and Zoller, 1991). Therefore, such a mutant enzyme should have no kinase activity. When radioactive ATP was added to the affinity purified wild type GST-Rim15, and phosphorylated proteins were visualized by SDS-PAGE and autoradiography, a 223 kDa band, the expected size of GST-Rim15, was identified on the autoradiogram (Figure 8A, lane 1). Some smaller labeled bands also appeared on the gel, probably resulting from proteolytic degradation of the full length GST-Rim15. The identity of the labeled bands as GST-Rim15 and fragments derived from this protein, as well as equal loading, were confirmed by immunoblot using anti-GST antibodies (not shown). The same phosphorylation experiment was repeated with the purified ATP-binding domain mutant GST-$Rim15^{K823Y}$. Only very weak phosphorylating activity (< 5%) was detectable, which could be due to a contaminating protein kinase co-precipitated with Rim15 (Figure 8A, lane 3). In a control experiment no labeling of GST-protein alone was observed (Figure 8A, lane 5). These results confirm that *in vitro* phosphorylation of the Rim15 protein results from an autocatalytic reaction, and, accordingly, that Rim15 is a protein kinase.

Analyses of epistasis as described above are consistent with a model in which Rim15 functions downstream and under negative control of cAPK in the Ras/cAMP pathway. It was therefore determined whether Rim15 and the ATP-binding site mutant $Rim15^{K823Y}$ could be phosphorylated by bovine cAPK (bovine A kinase catalytic subunit) *in vitro*. As can be seen in Figure 8A (lanes 2 and 4, respectively), both GST-Rim15 and to a lesser extent also GST-$Rim15^{K823Y}$ were strongly phosphorylated by cAPK, while no band appeared in the GST-control (lane 6). The somewhat lesser phosphorylation level observed for the ATP-binding domain mutant can probably be attributed to its lack of autophosphorylation. Commercially available bovine cAPK was used for practical reasons in these experiments because of the known instability of this enzyme. However, phosphorylation of Rim15 was also detected using a preparation of yeast Tpk1 expressed as an MBP-fusion protein in *E. coli* (Figure 9, lane 4). With these experiments it could be confirmed that Rim15 is a target of cAPK-phosphorylation *in vitro*.

As a next step it was investigated whether, as predicted by the working model, phosphorylation of Rim15 by cAPK inhibited its kinase activity, namely its ability to phosphorylate another protein. Since the endogenous substrates of Rim15 are not known, it was necessary to use an exogenous substrate, in this case α-casein. Commercially available casein consists of several isoforms. During the course of the experiments it was observed that Rim15 specifically phosphorylated the largest of the casein bands detectable

A
cAPK
GST-Rim1S
GST-Rim1S - K823Y
GST
relative units (%)
B
cAPK
cAPK Inhibitor
α-casein
GST-Rim1S
GST-Rim1S - K823Y
GST
relative units (%)
C
cAPK
α-casein
GST-Rim1S
GST-Rim1S- 5x (RRXS/A)
relative units (%)

Figure 8 (see last page): cAPK-dependent phosphorylation of Rim15 inhibits its kinase activity. **A.** GST-Rim15, GST-Rim15-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 were incubated with γ-[^{32}P] ATP either in the absence (-cAPK) or in the presence (+cAPK) of cAPK. Phosphorylation levels were quantitated by ^{32}P phosphoimager analysis and expressed as percent of the GST-Rim15-phosphorylation level (+cAPK). **B.** To analyze the effect of cAPK-dependent phosphorylation of Rim15, equal amounts of GST-Rim15, GST-Rim15-K823Y, and GST were preincubated with unlabelled 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-[^{32}P] ATP. The levels of α-casein phosphorylation were quantitated by ^{32}P phosphoimager analysis and expressed as percent of the control (level of α-casein phosphorylation after preincubation of GST-Rim15 in the absence of both cAPK and cAPK inhibitor). **C.** Equal amounts of GST-Rim15 and GST-Rim15-S709A/S1094A/S1416A/S1463A/S1661A (GST-Rim15-5x[RRXS/A], mutated at five consensus sites for cAPK-dependent phosphorylation, were analyzed as in B.

on an SDS-gel (see also Figure 9). By comparison with purified isoforms of α-, β-, and γ-casein this band was identified as α-casein (not shown). Rim15-dependent phosphorylation of α-casein was then studied after preincubation of GST-Rim15 in the absence or presence of bovine cAPK and/or A kinase inhibitor (Figure 8B). α-Casein was readily phosphorylated by wild-type Rim15 (lane 1). However, when preincubation of GST-Rim15 with cAPK was included in the assay protocol prior to the addition of α-casein, substrate phosphorylation was decreased by about 75% (lane 2). This inhibition of Rim15 activity could be counteracted by the addition of cAPK-specific inhibitor, confirming that it had been brought about by the activity of cAPK (lane 3). No phosphorylation of α-casein was detectable in the controls employing the ATP-binding site mutant allele of Rim15, confirming that this allele has no kinase activity (lanes 5-8). The activities observed were not due to contaminating kinase activity binding to the GST-tag or to the sepharose matrix as verified in the control with affinity-purified GST (lane 9). It should be pointed out that after incubation with cAPK Rim15 activity is not completely abolished, therefore it can be deduced that phosphorylation by cAPK may be not the only regulatory mechanism impinging upon Rim15.

The model that Rim15 could be regulated by cAPK had also been prompted by the presence of five consensus sites for cAPK-phosphorylation within the non-conserved amino acid sequence of Rim15. These sites might be decisive for the observed inhibition of Rim15 activity through phosphorylation by cAPK. To test this hypothesis, a mutant allele of Rim15 was constructed that had the conserved serine in all five consensus sites (Arg-Arg-X-Ser) changed to alanine. This mutant allele Rim15$^{S709A/S1094A/S1416A/S1463A/S1661A}$ was then assayed for its sensitivity to cAPK-phosphorylation *in vitro*. In Figure 8C it can be seen that the consensus site mutant (lanes 3, 4) in comparison to the wild type allele (lanes 1, 2) was not susceptible to inhibition by cAPK-phosphorylation.

Taken together, the biochemical evidence presented here supports the assumption that Rim15 is a protein kinase whose activity is under negative control of cAPK.

Tps1 is not a substrate for phosphorylation by Rim15 nor by cAPK in vitro

Initially Rim15 had been identified as an interactor of Tps1 in the two-hybrid system and it had been speculated whether Rim15 could be regulating Tps1 activity by phosphorylation. One argument supporting this assumption is the fact that *rim15Δ* strains are defective for trehalose accumulation in stationary phase and that in wild-type cells *RIM15* expression is enhanced in stationary phase. In order to find more direct proof for a regulatory role of Rim15 for trehalose synthesis, the ability of Rim15 to phosphorylate Tps1 *in vitro* was investigated. To this end, kinase assays with glutathione sepharose-bound GST-Rim15 (either truncated Rim15-P2 or the full length protein) and bacterially expressed MBP-Tps1 as substrate were performed. After SDS-PAGE analysis and autora-

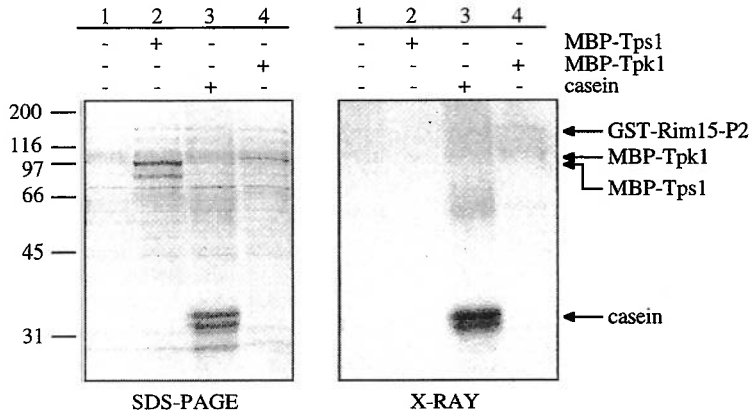

Figure 9: Tps1 is not phosphorylated by Rim15-P2. GST-Rim15-P2 was purified and analyzed for its autophosphorylation activity (lane 1), and its potential to phosphorylate either MBP-Tps1 (lane 2) or casein (lane 3) or to be phosphorylated by MBP-Tpk1 (lane 4). Equal amounts of GST-Rim15-P2 were incubated with γ-[^{32}P] ATP in the presence of either MBP-Tps1, MBP-Tpk1 or casein as described in *Material and Methods*. Proteins were separated by SDS-PAGE (left panel), and phosphorylation was detected by exposure to X-ray film (right panel).

diography, no phosphorylation of Tps1 by Rim15 was detectable, neither with the truncated GST-Rim15-P2 (Figure 9, lane 2), nor with the full length GST-Rim15 (Figure 10,

lane 1). Activity of Rim15 was verified in both cases by a control experiment including the exogenous substrate casein (or α-casein) in the reaction. As can be seen in the figures (Figure 9, lane 3; Figure 10, lane 2), this substrate was readily phosphorylated by the kinase preparations. In both cases, the presence of MBP-Tps1 (97 kDa band in lane 2), and α-casein (>31 kDa band in lane 3) was readily detectable on the coomassie-stained SDS-gels (left panels in Figures 9 and 10). This experiment was repeated several times, never detecting any phosphorylation of Tps1, independent of the fusion protein (GST-Tps1 or MBP-Tps1) used in the assays. Therefore, at present there is no biochemical evidence available to support the idea that Tps1 activity is regulated by Rim15-dependent phosphorylation. Still, it can not be ruled out completely that the *in vitro* differs from the *in vivo* situation. For instance, Tps1 was expressed as a heterologous protein in bacteria, this may result in altered properties of the enzyme and in unresponsiveness to phosphorylation by Rim15. However, at least GST-Tps1 had Tre6P synthase activity *in vitro* (results not shown).

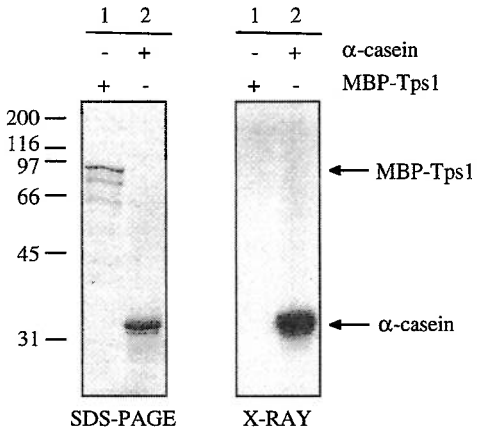

Figure 10: Tps1 is not phosphorylated by Rim15. Purified GST-Rim15 was analyzed for its potential to phosphorylate MBP-Tps1 (lane 1). Kinase activity of GST-Rim15 was verified by testing its potential to phosphorylate α-casein (lane 2). For the assays, equal amounts of purified glutathione-sepharose bound GST-Rim15 were incubated with γ-[^{32}P] ATP in the presence of either MBP-Tps1 or α-casein. Samples were centrifuged prior to boiling them with sample buffer in order to separate the glutathione-sepharose bound GST-Rim15 from the substrates (see *Material and Methods* for details). Supernatants were then subjected to SDS-PAGE (left panel) and phosphorylation was detected after exposure to X-ray film (right panel).

Expression of epitope-tagged Tps1 in *E. coli* has presented the opportunity to obtain pure native Tps1 without the other subunits of the Tre6P synthase/phosphatase complex. It was therefore for the first time possible to directly investigate the long-standing suggestion that Tps1 could be phosphorylated and thereby regulated by cAPK. GST-Tps1 was incubated together with bovine cAPK and γ-[^{32}P] ATP and then analyzed by gel electrophoresis and autoradiography. No phosphorylation of Tps1 was detectable (results not shown). Again the cautions raised above need to be considered, a bacterially expressed fusion protein may react differently from the endogenous protein. Also it is possible that bovine cAPK is not capable of phosphorylating Tps1 whereas cAPK from yeast would be. However, using bovine cAPK as a substitute for endogenous cAPK is a commonly accepted procedure in phosphorylation studies. Phosphorylation of Tps1 by cAPK is also unlikely since its amino acid sequence contains no consensus site for cAPK-phosphorylation (Arg-Arg-X-Ser). Interestingly also none of the other subunits of the Tre6P synthase/phosphatase complex contain this specific site. Taken together, it is very unlikely that Tps1 would be regulated by phosphorylation through cAPK *in vivo*. This finding is in agreement with the study of Vandercammen *et al.* (1989) who also detected no cAPK-dependent phosphorylation of Tps1.

DISCUSSION

Based upon the initial physiological analysis of a null mutant, it was proposed that Rim15 plays a critical role in a nutrient signaling pathway involved in the control of the transition to stationary phase. Moreover, it was suggested that the signaling pathway in question may be the Ras/cAMP pathway (see Chapter II). The results presented in the present chapter confirm and extend this hypothesis. They are most simply explained by a model in which Rim15 functions in the Ras/cAMP pathway downstream and under negative control of cAPK to regulate a number of physiological adaptations required for proper stationary phase entry. The following observations support this model.

(i) Deletion of *RIM15* suppressed the growth defect at elevated temperatures of a temperature-sensitive adenylate cyclase mutant (*cdc35-10*). Moreover, deletion of *RIM15* rendered cells independent of cAPK activity, demonstrated by the fact that a *tpk rim15 Δ* strain was viable. (ii) Overexpression of *RIM15* partially induced a starvation response in exponentially growing wild-type cells, complemented the defects in trehalose accumulation, in induction of *SSA3* expression, and in acquisition of thermotolerance of a *bcy1-1* mutant, and exacerbated the temperature-sensitive growth defect of strains compromised for cAPK activity (*cdc35-10*). (iii) Rim15 protein kinase activity was strongly inhibited by cAPK-dependent phosphorylation *in vitro*.

Some of the results described in the present chapter seem to be at variance with the above postulated model, because they revealed that the consequences of loss of *RIM15* were not completely identical to those of hyperactivity of cAPK. For instance, *RAS2^{val19}* and *bcy1* are known to result in complete inability to accumulate glycogen and to cause constitutive activation of trehalase and gluconeogenic growth defects, but this is not the case for *rim15Δ*. This contradiction can be reconciled with the model by assuming that cAPK constitutes a point of divergence in the Ras/cAMP pathway. This would acknowledge the fact that *rim15Δ* is epistatic to *tpk* with respect to some phenotypes (trehalose accumulation, induction of *SSA3*, *HSP12*, and *HSP26*, and thermotolerance) but not to all (glycogen accumulation and trehalase induction). The finding that *rim15Δ* cells contained low levels of glycogen in stationary phase may then seem contradictory. However, one possible explanation for this result would be that not only cAPK negatively controls Rim15 activity but that Rim15 in turn is involved in mechanisms of cAPK-feedback inhibition. This possibility should certainly be investigated in more detail in the future. One other interesting observation was made while studying the effects of loss of *RIM15* in a strain background with constitutively low, unregulated cAPK activity (*tpk^{w}*). In contrast to the *tpk rim15 Δ* strain, which accumulated very low levels of trehalose, did not acquire thermotolerance, was partially sensitive to starvation, and did not induce

expression of *SSA3*, *HSP12*, and *HSP26* in stationary phase, a *tpk^{w}rim15 Δ* strain accumulated high amounts of trehalose, was highly thermotolerant, insensitive to starvation, and able to partially induce expression of *SSA3, HSP12*, and *HSP26* under the same conditions (Table 2). Therefore, clearly *rim15 Δ* was not able to suppress *tpk^w* in the same way as complete loss of cAPK. This may indicate that unregulated cAPK (*i.e.* Tpk^w), when it is active during a stage where it would normally be inactivated by being bound to Bcy1 (*i.e.* stationary phase), is able to directly or indirectly (by activating an unidentified alternative pathway) interfere with targets usually under control of Rim15. Further studies will be necessary to corroborate this explanation.

Other pathways with partially overlapping or antagonistic functions with the Ras/cAMP pathway have been identified. The so-called main glucose repression pathway is the most intensively studied one of these pathways. A key role in this cAMP-independent pathway is played by the Snf1 protein kinase. Snf1 acts upon transcription factors such as the Mig1 repressor that prevents the expression of for instance gluconeogenic genes during growth on glucose (for a review see Ronne, 1995). But not all functions of Snf1 are exerted via Mig1 and it has been shown that Snf1 may mediate thermotolerance, starvation resistance, glycogen accumulation and proper G_1 arrest upon glucose exhaustion in a cAPK-antagonistic way (Thompson-Jaeger *et al.*, 1991; Timblin *et al.*, 1996; Huang *et al.*, 1996). In addition, two other genes coding for protein kinases have been identified through genetic screens for growth related effectors of cAPK, *YAK1* and *SCH9* (Toda *et al.*, 1988; Garrett and Broach, 1989). Overexpression of *SCH9* and deletion of *YAK1* are able to suppress the growth defect of strains lacking cAPK. Both of these kinases, however, are most likely elements of separate nutrient signaling pathways which are acting in parallel with the Ras/cAMP pathway (Garrett *et al.*, 1991; Denis and Audino, 1991; Hartley *et al.*, 1994). Formally it must be considered whether Rim15 could also act through one of these alternative pathways. Results presented in this chapter indicate that at least Rim15 and Yak1 are elements of separate pathways. Deletion of *YAK1* did not exacerbate the phenotype of a *rim15* strain (both in a strain with and without cAPK) for trehalose accumulation, as well as for thermo- and starvation tolerance and the two mutations did not have a synergistic effect. Rim15 could therefore in principle also act downstream of Yak1. Nevertheless, the fact that Rim15 can be phosphorylation-inactivated by cAPK *in vitro*, in addition with the results of the analyses of epistasis with the Ras/cAMP pathway strongly supports the idea that Rim15 constitutes the first protein kinase being a downstream effector of cAPK in *S. cerevisiae*.

Current knowledge indicates that Rim15 may further be under regulation by cAPK-independent mechanisms. This is underlined by two observations. First, Rim15 was not fully inhibited by cAPK-phosphorylation *in vitro*. Second, overexpression of the cAPK-unresponsive *rim15$^{5x(RRXS/A)}$* mutant gene compared with overexpression of the wild-type gene, caused no additional effects in exponentially growing cells (data not shown).

However, this latter observation could also be explained by assuming that the level of overexpression in log-phase cells is so high that already the increased level of wild-type Rim15 is sufficient to escape downregulation by cAPK and expression of a putatively overactive allele has therefore no additional effects. This explanation seems plausible since it is known that during growth on glucose only very low amounts of *RIM15* mRNA and Rim15 protein are present in the cells (Vidan and Mitchell, 1997; this study). However, the relatively moderate effects caused by *RIM15* overexpression in exponentially growing cells, make it also likely that other posttranscriptional or posttranslational factors influence Rim15 activity. These could downregulate Rim15 protein levels in log-phase, for instance by mRNA degradation or proteolysis. It is also possible that specific activating elements of Rim15 are lacking in cells growing on glucose. Also, active cAPK may relieve some of the consequences of *RIM15* overexpression in an indirect manner (*e. g.* through activation of trehalase). It should be expected that the full induction of stationary phase adaptations depends upon a number of signaling events that have to be integrated in order to yield the response. Strong overactivity of one element, *e.g.* Rim15, would not and should not be sufficient to override the input from other control elements, this way providing some sort of insurance system for the cells.

Another important question raised in this chapter, was whether Tps1 may be a direct target of Rim15. This idea is supported by two lines of evidence. First, Tps1 and Rim15 interacted in a two-hybrid analysis. Second, as it has already been pointed out in the Discussion of Chapter II, the observed lack of induction of trehalose accumulation in stationary phase *rim15Δ* strains suggests Rim15-dependent posttranslational Tps1-regulation. This suggestion is supported by the finding that even though *rim15Δ* cells had normal levels of the enzymes responsible for trehalose synthesis (Tre6P synthase and Tre6P phosphatase), they were virtually unable to accumulate trehalose *in vivo*. Remarkably, trehalose accumulation in stationary phase was largely independent of the presence of cAPK but dependent upon Rim15. This can be concluded from the observation that in stationary phase a *tpk yak1* strain contained very high levels of trehalose while a *tpk rim15 Δ* strain accumulated very little trehalose (Table 1).

The trehalose content of yeast cells is correlated with the activity of the Ras/cAMP pathway: high activity of cAPK coincides with low levels of trehalose, low cAPK activity coincides with high levels of trehalose (Toda *et al.*, 1985; Hottiger *et al.*, 1989; see also General Introduction). Therefore it had been speculated earlier that both the catabolism and the synthesis of trehalose could be regulated by the Ras/cAMP pathway in an antagonistic manner (Panek *et al.*, 1987, see also General Introduction). While neutral trehalase has been shown to be phosphorylation-activated *in vitro* by cAPK, the evidence presented for a direct regulation of Tre6P synthase by cAPK-phosphorylation has been doubtful (Uno *et al.*, 1983; Panek *et al.*, 1987; Vandercammen *et al.*, 1989). Also in the present study, no *in vitro* phosphorylation of bacterially expressed, purified Tps1 could

be detected after incubation in the presence of cAPK. However, trehalose synthesis may still be controlled through the Ras/cAMP pathway, not by phosphorylation-*inactivation* of Tre6P synthase by cAPK but instead by phosphorylation-*activation* by Rim15. In accordance with such a model, it could then be predicted that hyperactive cAPK (*e.g.* in a *bcy1* mutant) would prevent trehalose accumulation not only by constantly activating trehalase but also by inhibiting Rim15, and consequently Tre6P synthase activity. A strain with low cAPK activity would then have high levels of trehalose because in addition to failing to activate trehalase, it would also not, or to a lesser degree restrict Rim15 activity and thereby stimulate trehalose synthesis. Accordingly, overexpression of Rim15 induces trehalose synthesis to some extent in exponentially growing wild-type cells (which have active cAPK), and suppresses the lack of trehalose accumulation in a *bcy1-1* strain in stationary phase, despite the presence of constantly active neutral trehalase.

The exact influence of Rim15 on trehalose synthesis remains to be elucidated. No phosphorylation of Tps1 by Rim15 was detected *in vitro*. This could be due to the fact that both proteins were expressed as fusion proteins and may therefore have altered biochemical properties. Other biochemical data to corroborate the results from the initial two-hybrid analysis are currently not available. The coprecipitation studies detected physical interaction between Tps1 and Rim15 but the interpretation of this result remains difficult, since it could not be resolved from other, clearly unspecific interactions. Future studies should try to resolve the technical problems encountered in these experiments in order to make a clear-cut statement.

One further possibility needs to be taken in account. The detected weak two-hybrid interaction between Tps1 and Rim15 may well signify a more indirect action of Rim15. It could be speculated that Rim15 binds to Tps1 but phosphorylates other parts of the Tre6P synthase/phosphatase complex and influences trehalose synthesis in this way. Likely candidates for phosphorylation by Rim15 are the two large subunits, Tps3 and Tsl1. Not only have they been implied in having regulatory functions, but they have also found to be highly homologous to each other (Vuorio *et al.*, 1993; Ferreira *et al.*, 1996; Reinders *et al.*, 1997; Bell *et al.*, 1998). This makes it likely that they contain sites specific for Rim15-dependent phosphorylation found both in Tps3 and Tsl1, but not in the other subunits.

One of the consequences of loss of *RIM15* was a severe reduction of sporulation (see *Chapter II*), a phenotype that is also found in strains with hyperactive cAPK. It has been a long-standing suggestion that the Ras/cAMP pathway exerts nutritional control over the initiation of meiosis (Matsumoto *et al.*, 1983a), but the exact nature of this control has not been unraveled. A recent publication now described the identification of Rim15 as a positive regulator of the expression of early meiotic genes and of the induction of sporulation (Vidan and Mitchell, 1997). Consequently, another exciting outcome

of this study is the possibility that Rim15 may confer the missing link between the Ras/cAMP pathway and early meiotic gene expression.

GENERAL DISCUSSION

Trehalose metabolism in *S. cerevisiae* has received growing interest in recent years for two reasons: First, it was found that the accumulation of trehalose is part of the stress response of yeast and that accumulation of this metabolite renders cells more resistant to the adverse effects of various forms of stresses, such as heat or desiccation (Van Laere, 1989; Wiemken, 1990). By studying the accumulation of trehalose in response to stress and comparing the contribution of this factor to the acquisition of stress tolerance (thermotolerance) with the contributions of other factors like hsps, much has been learned about the strategies by which yeast survives adverse conditions. Second, the key enzymes of trehalose metabolism have been shown to be regulated by and involved in important signaling pathways in *S. cerevisiae*. While the trehalose degrading enzyme neutral trehalase has been shown to be activated through phosphorylation by cAPK *in vitro* (Uno *et al.*, 1983; App and Holzer, 1989), the Tre6P synthase seems to have an important role besides trehalose synthesis in regulating the glycolytic flux (Van Aelst *et al.*, 1993; Bell *et al.*, 1992; Thevelein and Hohmann, 1995). Studies of the regulation of neutral trehalase by phosphorylation, have resulted in considerable progress in the understanding of the mechanisms of nutrient-induced signal transduction. It may therefore be expected that studies of the regulation of Tre6P synthase may also broaden our general understanding of nutrient signaling processes in yeast, in particular because of the unexpected and still not fully understood role of the Tre6P synthase in the regulation of glycolysis.

Because the observed pattern of trehalose accumulation, which was found to be negatively correlated with the growth rate, predicts a tight regulation of the involved enzymes, many groups have tried to unravel the underlying mechanisms. Despite the concerted efforts of several labs, the exact factors regulating the activity of Tre6P synthase and thereby the accumulation of trehalose, have not been unequivocally identified (see Introduction of Chapter II for details). Therefore, in the present thesis two different approaches have been used directed at elucidating the regulatory processes of trehalose synthesis in *S. cerevisiae*. In a first approach (Chapter I), structure and function of the Tre6P synthase/phosphatase complex and of its subunits under the conditions of a sublethal heat shock were studied in detail. In a second approach (Chapter II), the yeast two-hybrid system was employed in order to identify potential regulators of Tps1, the Tre6P synthase. This second approach led to the isolation of a gene coding for a new protein kinase, Rim15. Results obtained so far suggest that Rim15 is a regulator of trehalose synthesis in *S. cerevisiae*. Moreover, it could be shown that Rim15 activity is negatively regulated by cAPK, the central enzyme of the Ras/cAMP pathway. This important nutrient signaling pathway has been suggested previously to regulate the trehalose content of

yeast cells through the activation of neutral trehalase and/or inactivation of Tre6P synthase/phosphatase (Uno *et al.*, 1983; Panek *et al.*, 1987; François *et al.*, 1991; Thevelein, 1996).

Who is who in trehalose synthesis - the roles of Tps1, Tps2, Tps3, and Tsl1

Despite the fact that the basic mechanism of trehalose synthesis in *S. cerevisiae* has already been clarified 40 years ago (Cabib and Leloir, 1958), the actual identification of the enzymes catalyzing this process has only been achieved recently and with the aid of molecular biology (Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Vuorio *et al.*, 1993; McDougall *et al.*, 1993). Analyses by classical biochemical methods had been rendered difficult by the fact that Tre6P synthase and Tre6P phosphatase purified as parts of a large multimeric protein complex, which was very sensitive to proteolytic degradation during purification (Vandercammen *et al.*, 1989; Londesborough and Vuorio 1991; Bell *et al.*, 1992; De Virgilio *et al.*, 1993). The systematic approach using deletion mutants and the two-hybrid system, as presented in this thesis, now allowed us to assess the roles of the single subunits both *in vivo* and *in vitro*, making it finally possible to settle some time-honored disputes (see Chapter I).

The identity of Tps1 with the Tre6P synthase had been questioned because of the surprising finding that *TPS1* is allelic to a number of known mutations believed to be involved in the regulation of glycolytic flux (Gonzáles *et al.*, 1992; Bell *et al.*, 1992; Van Aelst *et al.*, 1993). Here we have demonstrated convincingly that Tps1 has Tre6P synthase activity *in vitro* as well as *in vivo*. Moreover, it was also shown that none of the other subunits has Tre6P synthase activity, despite the extensive sequence homologies of *TPS2*, *TPS3*, and *TSL1* with *TPS1*. The biological significance of these homologies remains to be investigated, but it has been suggested that they may have a role in the oligomerization of the complex (Thevelein and Hohmann, 1995).

The identity of Tps2 with the Tre6P phosphatase has not been seriously challenged, since deletion of *TPS2* caused yeast cells to accumulate high amounts of Tre6P instead of trehalose during heat shock and in stationary phase (De Virgilio *et al.*, 1993). In fact, Londesborough and Vuorio (1993) have been able to separate the 102 kDa subunit, later identified as Tps2, from the complex and have shown that it has Tre6P phosphatase activity. We could confirm that Tps2 is the Tre6P phosphatase by showing that its deletion always caused accumulation of Tre6P under condition of heat shock as long as cells contained an intact Tps1. The fact that the *in vitro* activity of the Tre6P phosphatase was strongly affected by *tps1Δ*, but virtually unaffected by *tps3Δ* or *tsl1Δ*, indicates that Tps2 may need binding to Tps1 for its optimal activity.

While the presence of Tsl1 in the complex is known from enzyme purification, the possibility of Tps3 being a fourth subunit of the complex has only been discussed on the basis of its homologies with Tsl1 (Vuorio *et al.*, 1993; Thevelein and Hohmann, 1995). Now we were able for the first time to present evidence that Tps3 is indeed a functional component of the Tre6P synthase/phosphatase complex, by showing that it not only binds to Tps1 and Tps2 in a two-hybrid assay but also that its deletion in a *tsl1Δ* background seriously affected trehalose accumulation during heat shock. Moreover, in collaboration with the group of Thevelein we were also able to demonstrate that the loss of both large subunits seriously destabilized the binding between Tps1 and Tps2 (as seen by the Superose 6-FPLC elution profile and subsequent immunoblot analysis; Bell *et al.*, 1998). Besides this function in conferring the structural integrity of the complex, Tps3 and Tsl1 also affect the susceptibility of Tps1 to inhibition by phosphate. Earlier, Vuorio *et al.* (1993) concluded that Tsl1 is responsible for mediating the observed allosteric effects of Fru6P and phosphate on the Tre6P synthase activity since these effects were lost by partial truncation of Tsl1 *in vitro*. Here we found that *tps3Δ* alone had no influence on phosphate inhibition of Tre6P synthase, *tsl1Δ* alone diminished this inhibiting effect, and a *tps3Δ tsl1Δ* strain combined converted the inhibitory effect into an activatory effect. The implications of these observations for the role of Tps1 in glycolysis are discussed below (Bell *et al.*, 1998, my own results, not shown). In another recent study, a *tsl1Δ* or a *tsl1Δ tps3Δ*, but not a *tps3Δ* strain, were shown to be affected in their ability to induce Tre6P synthase activity upon stationary phase entry (Ferreira *et al.*, 1996), indicating a particular role for Tsl1 in stationary phase. In accordance with this notion, *TSL1* expression, unlike *TPS1*, *TPS2*, and *TPS3* expression, was found to be strongly enhanced in stationary phase (Winderickx *et al.*, 1996). This may also account for the fact that only Tsl1 protein has been purified in earlier studies, since these purifications were generally performed from stationary phase cells (*e.g.* Bell *et al.*, 1992). Taken together, is clear that despite the observed homologies and the functional overlaps, Tsl1 and Tps3 have very distinct roles which depend on the physiological conditions.

The dual role of Tps1- new insights

Our detailed studies of the various deletion mutants of the subunits of the Tre6P synthase/phosphatase complex have confirmed that Tps1 has a unique role during growth on rapidly fermentable carbon sources. Deletion of none of the other subunits caused a glucose-negative phenotype similar to the one conferred by *tps1Δ*. Also, this defect was not suppressed by deletion or overexpression of any of the other subunits (Bell *et al.*, 1998; my own results, not shown). The putatively dual role of Tps1 both as Tre6P synthase and as a regulator of glycolysis has been a puzzle to many groups over the years.

Three models have been proposed to explain the observed pleiotropic phenotype of a *tps1Δ* strain, namely (i) the 'general glucose sensor' model, (ii) the phosphate recovery hypothesis, and (iii) the hexokinase inhibition model (Thevelein and Hohmann, 1995; see also General Introduction for details). Thevelein and Hohmann (1995) have already pointed out that model (i) and (iii) could be easily merged by assuming that Tps1 associates with hexokinases and that it exerts its proposed regulatory effects by inhibiting these enzymes via synthesized Tre6P. In the light of new insights resulting from this and other recent studies, this idea seems very attractive. In the following section the conceptual advantages of such a combined model and the evidence supporting it will be discussed briefly:

Our findings and the results of our collaborators indicate that Tps1 can function without the other subunits present and that it occurs as a free subunit in wild-type cells (this study, Bell *et al.*, 1998). This is one prerequisite of the proposed model. The finding that the free Tps1 was stimulated instead of inhibited by phosphate, underscores the assumption that it has a function that is different from the complex-bound Tps1 (Bell *et al.*, 1998). It also indicates that free Tps1 may be active when the Tre6P synthase/phosphatase complex is not. This in turn solves the problem that Tre6P synthase activity is downregulated upon glucose addition (François *et al.*, 1991), because free Tps1 may not be affected in the same way. It has been observed that at the onset of fermentation wild-type cells contain high phosphate concentrations, which would stimulate the activity of the free Tps1. Accordingly, as metabolism becomes adjusted to fermentation, and phosphate levels start to decline Tre6P-mediated hexokinase inhibition would be reduced by downregulation of the activity of free Tps1 (Van Aelst *et al.*, 1993). In this context, it has been shown that very little Tre6P synthase activity was sufficient to overcome the effect of *tps1Δ* (about 10% of the wild-type level; Hohmann *et al.*, 1994). This can also be explained if not trehalose synthesis is needed to recover phosphate, but Tre6P synthesized by Tps1 in close association with hexokinases. Finally, this proposed model can also resolve two conceptual problems of earlier explanations. First, no intensive futile cycling of trehalose needs to be proposed, since not the Tps1 in the Tre6P synthase/phosphatase complex but the free Tps1 would be active. Second, the fact that no or very little Tre6P can be detected in wild-type cells growing on glucose (Hohmann *et al.*, 1996) is no contradiction to inhibition of hexokinases *in vivo* by this metabolite, since the free Tps1 is expected to associate with the hexokinases directly and, therefore, probably a very high Tre6P concentration can be reached locally. In spite of the attractiveness of the proposed model, at the moment no evidence for a direct binding of Tps1 to hexokinases has been demonstrated, which is undoubtedly a main drawback. However, it should be relatively easy to test the proposed interaction by means of the two-hybrid system.

Inhibition of *S. cerevisiae* hexokinases by Tre6P has been demonstrated *in vitro* (Blázquez *et al.*, 1993). In addition, a model in which Tps1 restricts hexokinases through Tre6P is also indirectly supported through results obtained from other fungi. Accordingly, it was found that the *in vitro* sensitivity of hexokinases towards Tre6P correlated well with the *in vivo* effect of *TPS1* deletion. For instance, hexokinases in *S. pombe* were found to be inhibited *in vitro* by Glu6P (like in mammalian cells) but, unlike in *S. cerevisiae,* not by Tre6P and the corresponding *tps1* mutant was not defective for growth on glucose (Blázquez *et al.*, 1994). Similarly, hexokinases in *A. niger* were found to be weakly inhibited *in vitro* by Tre6P and the corresponding Tre6P synthase mutant (*tpsA*) mutant was only weakly defective for growth on glucose (Arisan-Atac *et al.*, 1996; Wolschek and Kubicek, 1997).

Most interestingly, recent results suggest that a similar control mechanism involving low amounts of Tre6P for the regulation of sugar influx into glycolysis might also exist in higher plants. This idea is supported by the following line of evidence: Accumulation of relatively low amounts of trehalose in transgenic tobacco plants overexpressing the Tre6P synthase and Tre6P phosphatase genes from *E. coli* (*otsA* and *otsB*) cause strong morphological effects (*e.g.* stunted growth, lancet-shaped leaves), which might indicate that Tre6P has a function as a signal molecule in plants (Goddijn *et al.*, 1997). This is further corroborated by the finding that plants contain homologs of *TPS1* and *TPS2*, and that they synthesize low amounts of trehalose when treated with the trehalase inhibitor validamycin (Goddijn *et al.*, 1997; Blázquez *et al.*, 1998; Vogel *et al.*, 1998). This in turn indicates that trehalose synthesis may occur naturally in plants, even though the amounts accumulated may be so low that they escape detection under normal circumstances. Since plant hexokinases have been indicated to play a central role in sugar sensing and in the regulation of sugar induced gene expression, it will be highly interesting to determine whether they could be regulated by Tre6P similar to the yeast model (for reviews see Jang and Sheen, 1994; Smeekens and Rook, 1997). Clearly more work needs to be done to verify this suggested regulatory mechanism in plants. Should it hold true, it would be yet another impressive example for the power of elucidating signaling pathways in *S. cerevisiae* and applying the findings to more complex organisms.

The regulation of trehalose synthesis and RIM15

One of the aims of the present Ph.D. thesis was the identification of potential regulating proteins of Tre6P synthase. The existence of posttranslational regulation mechanisms for the induction of Tre6P synthase activity was predicted by earlier results, for instance by the finding that inhibition of protein synthesis during a heat-shock treatment only partially reduced the activation of Tre6P synthase *in vitro* as well as the resulting ac-

cumulation of trehalose *in vivo* (Hottiger, 1988; De Virgilio *et al.*, 1991b). Interestingly, by means of two-hybrid analysis we have identified a new gene which encodes a protein kinase (Rim15), whose deletion results in a highly pleiotropic phenotype, including a defect in trehalose accumulation in stationary phase. Even though the exact mechanism by which Rim15 influences trehalose accumulation in stationary phase has not been fully clarified (see below), it is still possible to draw some basic conclusions on the regulation of trehalose synthesis from the results presented in Chapters II and III. One conclusion is that different regulatory mechanisms influence trehalose accumulation in stationary phase and under heat-shock conditions. Trehalose accumulation in stationary phase was shown to be dependent on Rim15, since *rim15Δ* cells failed to accumulate trehalose when glucose was depleted from the medium. However, the same *rim15Δ* cells were not impaired in their ability to induce trehalose synthesis in response to a mild heat shock, neither in stationary nor in log-phase. Based on the finding that *RIM15* is only weakly expressed in cells growing on glucose, and that Rim15 protein is virtually absent from these cells it is not surprising that *rim15Δ* did not cause any notable effect on trehalose synthesis in log-phase cells (Vidan and Mitchell, 1997; this study).

A comparison of trehalose synthases between *S. cerevisiae* and other fungal species reveals that some fungi, such as *A. niger* and *C. utilis* possess two differently regulated Tre6P synthases (Vicente-Soler *et al.*, 1989, 1991; Wolschek and Kubicek, 1997). In the case of *A. niger,* transcription of one of these Tre6P synthases (*tpsB*) is strongly enhanced during heat shock, while the other one (*tpsA*) is constitutively expressed, indicating that in this fungus two enzymes are performing the function of Tps1 in *S. cerevisiae*. It is therefore not surprising to find that in *S. cerevisiae* Tre6P synthase is controlled by at least two different mechanisms, one which regulates the response to sudden stresses such as a mild heat shock, and another which regulates the response to slow nutrient depletion.

The two-hybrid data suggest a direct interaction between Tps1 and Rim15, and the observed effects of *rim15Δ* upon trehalose accumulation during stationary phase entry support this idea. However, so far no further evidence for a direct regulation of Tre6P synthase activity by Rim15, *i.e.* by phosphorylation, could be obtained. While this may easily be due to the conditions chosen for these assays (as pointed out in Chapter III), it could also be possible that Rim15 binds to Tps1 but phosphorylates one of the other subunits of the complex. Another possibility is that the detected binding of Rim15 and Tps1 in the two-hybrid system was mediated by a third protein, that functioned as a 'bridge' between them. Again, this hypothetical third protein could be a component of the Tre6P synthase/phosphatase complex. The recent findings on the importance of Tsl1 and Tps3 for the structure and function of the Tre6P synthase/phosphatase complex (see above) make it tempting to speculate that these subunits, especially Tsl1, may be direct targets of Rim15 (this study; Bell *et al.*, 1998). This possibility should therefore be examined in future studies.

Regulation of trehalose metabolism in *S. cerevisiae* has been linked to the activity of the Ras/cAMP pathway before. The idea of a direct influence of this pathway on trehalose accumulation was strongly supported by the demonstrated cAPK-dependent phosphorylation of neutral trehalase *in vitro* (Uno *et al.*, 1983; App and Holzer, 1989). We have not been able to find evidence for the long-standing suggestion that cAPK directly regulates Tre6P synthase by phosphorylation (no phosphorylation of isolated Tps1 was detectable after incubation with cAPK). However, since Rim15 was found to be a downstream effector of cAPK in the Ras/cAMP pathway (see below), it may be possible that the induction of trehalose synthesis upon stationary phase entry is controlled through this pathway via Rim15. How exactly Rim15 regulates the activity of the Tre6P synthase/phosphatase complex on a molecular basis still remains to be elucidated, as has been pointed out above.

The role of Rim15 in stationary phase entry

Deletion of *RIM15* was shown to confer a pleiotropic phenotype in cells that were starved for glucose (and to a lesser extent if starved for nitrogen). In addition to the described defect in trehalose accumulation, this phenotype also included the failure to accumulate glycogen, to arrest properly in G_1, to induce expression of *SSA3, HSP12*, and *HSP26* (genes under negative control of cAPK), and to acquire thermo- as well as starvation tolerance. These findings strongly suggest that Rim15 may be part of a nutrient signaling pathway and that loss of its function prevents the cells from entering stationary phase properly. Since the phenotype of a *rim15Δ* strain was similar to the phenotypes conferred by hyperactivity of cAPK (*e.g.* by *bcy1*), it was further investigated whether Rim15 could have a role in the Ras/cAMP pathway. Results obtained from analyses of epistasis and kinase assays with Rim15 and cAPK *in vitro* are most simply explained with a model in which Rim15 acts downstream of and under negative control of cAPK to control stationary phase entry. In the following paragraphs the suggested model (see Figure 1) and the evidence supporting it will be explained in more detail:

Several lines of evidence suggest that log-phase cells growing on glucose have only very low levels of Rim15 activity *in vivo*: First, *rim15Δ* cells had no obvious phenotype in cells growing on glucose. Second, *RIM15* itself has been shown to be under glucose-repression (Vidan and Mitchell, 1997; this study). Third, based on our finding that Rim15 activity is inhibited by cAPK phosphorylation *in vitro* and on the fact that cAPK is highly active in log-phase cells, Rim15 activity is expected to be downregulated in these cells. Fourth, preparations of GST-Rim15 from log-phase cells yielded always largely degraded GST-Rim15 protein, while preparation from stationary phase cells yielded stable GST-Rim15 protein (this study, data not shown, see also Vidan and Mitchell, 1997;

see also below). Fifth, overexpression of a potentially cAPK-unresponsive *RIM15*-allele (*rim15*$^{5x(RRXS/A)}$) induced the same partial starvation response in wild-type cells during log-phase as overexpression of *RIM15* suggesting the presence of yet unidentified post-translational regulatory mechanisms.

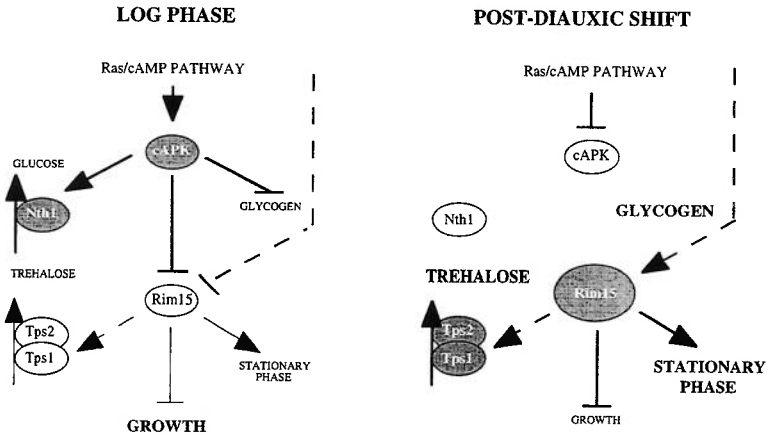

Figure 1: Model of the cAMP-dependent protein kinase (cAPK) - Rim15 pathway. Glucose-repression of *RIM15* as well as posttranslational inhibition via cAPK-dependent phosphorylation, and potentially an additional yet to be identified mechanism, results in low Rim15 kinase activity in log phase. During the post-diauxic shift, glucose derepression of *RIM15* (illustrated by the increase in size of the Rim15 protein), downregulation of cAPK-dependent inhibition, and potential activation by yet to be identified mechanisms result in high Rim15 kinase activity. Rim15 acts as a positive regulator of stationary phase entry and, consequently, as a negative regulator of growth. An arrow indicates a positive interaction, a bar indicates a negative one. 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, active proteins on shaded background. Nth1, Tps1, and Tps2 denote neutral trehalase, Tre6P synthase, and Tre6P phosphatase, respectively.

During the diauxic shift, when glucose is depleted from the growth medium and the cAMP level drops, cAPK becomes inhibited, through binding to the regulatory subunit Bcy1. Consequently, inhibition of Rim15 is relieved and Rim15 can induce a number of adaptations necessary for proper stationary phase entry. These include arrest in G_1, accumulation of trehalose, acquisition of thermotolerance and expression of *SSA3*, *HSP12*, and *HSP26*. The following results support this part of the model: First, deletion of

RIM15 suppressed the effects of mutations that inactivate cAPK (*cdc35-10*, *tpk*; this study, Chapter III). Second, overexpression of *RIM15* exacerbated the effects of mutations that inactivate cAPK (*cdc35-10*; this study, Chapter III) and suppressed the effects of mutations that constitutively activate cAPK (*bcy1-1*; this study, Chapter III). Third, a *rim15Δ* strain failed to arrest properly in G_1 (this study, Chapter II), indicating that growth arrest upon stationary phase entry depends, at least partially, upon Rim15.

Nevertheless, not all responses to nutrient starvation are mediated via Rim15, which was shown by the fact that *rim15Δ* suppressed some but not all phenotypes of a strain without functional cAPK (this study, Chapter III). While a *tpk rim15Δ* strain failed to accumulate trehalose, did not induce expression of *SSA3*, *HSP12*, and *HSP26*, and was thermosensitive, it still hyperaccumulated glycogen and was only partially sensitive to starvation. Consequently, we suggest that Rim15 constitutes a branching point in the Ras/cAMP pathway. Further, Rim15-activating mechanisms are proposed, since overexpression of *RIM15* in exponentially growing cells only partially induced a starvation response (as seen by the induction of trehalose synthesis, *SSA3*-induction and acquisition of thermotolerance), which may indicate that these Rim15-activating factors are absent from cell growing on glucose (and/or that Rim15 inactivating factors are present, see above). Such an activating factor could for instance be a protein phosphatase. Elucidating the role and identity of these additional factors impinging on Rim15 should be one of the goals in the future.

At this point it should also be noted, that our model indirectly predicts that inhibition of Rim15 activity should be a necessary step for cells reentering mitosis. In support of this model, it has recently been shown that Ras protein activity is required for spore germination in yeast, in order to initiate reentry into the cells cycle. Temperature-sensitive mutants that deactivate the Ras/cAMP pathway (*cdc25-1*, *ras2-23*, and *cdc35-1*) were unable to germinate at the nonpermissive temperature (Herman and Rine, 1997). In accordance with our model, these phenotypes can be interpreted as a consequence of the failure of the cells to inactivate Rim15.

Does Rim15 have a role in a general stress response ?

The Ras/cAMP pathway is also involved in the general stress response of *S. cerevisiae*. Yeast cells with constitutively active cAPK (*bcy1*) fail to acquire thermotolerance in response to a mild heat shock, while cells with reduced cAPK activity (*cyr1-2*) constitutively synthesize some heat shock proteins and are thermotolerant (Shin *et al.*, 1987). Many genes that are induced during stress response have been shown to be under negative control of cAPK, for instance *SSA3*, *CTT1*, and *HSP12* (Boorstein and Craig, 1990b; Praekelt and Meacock, 1990; Wieser *et al.*, 1990). These genes have in common

that their promoters contain a cAMP-responsive element, also called STRE (Marchler *et al.*, 1993). Recently, it has been demonstrated that gene expression in response to many stresses may be conferred by the Msn2/Msn4 transcription factors via STREs (Martinez-Pastor *et al.*, 1996). During exponential growth cAPK activity is high and therefore inhibits the induction of a stress response. Consequently, it has been suggested that during adverse growth conditions cAPK activity would be lowered by yet unknown mechanisms which would then allow the cell to switch from growth to stress response (Siderius and Mager, 1997). From what we know, Rim15 seems to play a minor role in this general stress response in log-phase cells, which can also be explained by *RIM15* being under (partial) glucose repression (Vidan and Mitchell, 1997; this study). Accordingly, no effect of *rim15Δ* was detected in heat-shocked cells with respect to trehalose accumulation or the acquisition of thermotolerance.

Possible relationship of Rim15 to other nutrient signaling pathways

Other pathways with partially overlapping or antagonistic functions with the Ras/cAMP pathway have been identified. The best studied one of these alternative pathways is the main glucose repression pathway. Like the Ras/cAMP pathway it is involved in regulating the metabolic switch from fermentative to gluconeogenic growth. In contrast to the Ras/cAMP pathway, the main glucose repression pathway is regulated by the Snf1 protein kinase and does not involve cAMP as a second messenger. The role of Snf1 in glucose repression can be largely explained by its negative control of transcription factors involving DNA-binding proteins such as Mig1 (for a review, see Ronne, 1995). However, not all functions of Snf1 are mediated by Mig1, and it has recently been suggested that Snf1 may have functions which may partially overlap with the ones of the Ras/cAMP pathway. Accordingly Snf1 was found to control thermotolerance, starvation resistance, glycogen accumulation and proper G_1 arrest upon glucose exhaustion in a cAPK-antagonistic way (Thompson-Jaeger *et al.*, 1991; Timblin *et al.*, 1996; Huang *et al.*, 1996). Two other genes with significant homology to protein kinases, *YAK1* and *SCH9* have been isolated in screens for growth-related effectors of cAPK (Toda *et al.*, 1988; Garret and Broach, 1989). Current knowledge suggests that their corresponding gene products confer separate nutrient signaling pathways, acting in parallel with the Ras/cAMP pathway (Toda *et al.*, 1988; Garrett *et al.*, 1991; Thompson-Jaeger *et al.*, 1991; Hartley *et al.*, 1994).

Other recently discovered pathways that may be involved in nutrient signaling and growth control are the Tor pathway and the Whi2 pathway. The central elements of the Tor pathway are the two homologous phosphatidylinositol protein kinases Tor1 and Tor2. Tor seems to be required to signal the availability of nutrients and consequently a

lack of this signal results in growth arrest and stationary phase. Therefore, loss of Tor triggers a cellular response that is reminiscent of cells entering stationary phase, for instance arrest in early G1, severe reduction of protein synthesis, hyperaccumulation of glycogen, acquisition of thermotolerance, enlargement of the vacuole, and the transcriptional induction or repression of certain genes (Hall, 1995; Barbet *et al.*, 1996). The same kind of response is also conferred by treatment of wild-type cells with rapamycin, while TOR mutants are insensitive to rapamycin. Even though the mode of action of Tor has not been clarified in detail, it could be demonstrated that the primary role of Tor seems to be to control general protein synthesis and thereby general growth. The G_1 arrest observed in *tor* mutants would then be a secondary effect of the lack of protein synthesis needed at all stages of the cell cycle but especially during G_1 progression (Barbet *et al.*, 1996). The effects of loss of Tor function are similar to the effects that we observed in cells overexpressing *RIM15*. Consequently, while Tor activates growth by signaling the presence of nutrients, Rim15 inactivates growth by signaling the absence of nutrients. It is therefore possible that Tor and Rim15 act in an antagonistic way in order to prevent early G_1 progression and growth in the absence of nutrients. However, Tor seems not to act via the Ras/cAMP pathway (Barbet *et al.*, 1996) and preliminary results (data not shown) contradict the idea that Tor and Rim15 are controlled by the same pathway.

The *WHI2* gene codes for another important protein, mediating the coordination of growth and cell proliferation. *Whi2* mutant cells behave like wild-type cells in exponential phase, but develop a pleiotropic phenotype upon entry into stationary phase. The traits found in starved *whi2* cells include continued division even upon carbon source exhaustion, abnormally small cell size, higher cell density, random arrest in the cell cycle, failure to acquire stress tolerance, lack of glycogen accumulation, and rapid loss of viability. In summary, *whi2* cells retain properties of exponentially growing cells in the absence of nutrients (Sudbery *et al.*, 1980; Saul *et al.*, 1985). The described phenotype of a *whi2* mutant is remarkably similar to the phenotype of a *rim15Δ* strain. Interestingly, *WHI2* mRNA is only present during the exponential phase but not during the diauxic shift (Mountain and Sudbery, 1990). Its expression is therefore inversely regulated with *RIM15* expression. However, Whi2 has not been associated with any other known regulatory pathway and no further elements interacting with Whi2 are currently known.

Taken together, several pathways have been identified that are involved in nutrient signaling in *S. cerevisiae*. Even though we have convincing evidence that Rim15 acts through the Ras/cAMP pathway, it still formally needs to be considered whether Rim15 may also be involved in one of the other pathways. Preliminary results obtained so far have not indicated an involvement of Rim15 in any of these alternative pathways. However, these pathways are generally not very well characterized which renders studying them rather

difficult and often makes results hard to interpret. As more results become available in the future it should be easier to connect the single pieces and to draw a more complete picture.

Rim15 - the missing link between meiosis and the Ras/cAMP pathway ?

Diploid yeast cells that are starved for nitrogen in the absence of glucose are able to initiate meiosis and subsequently sporulate. The starvation signal that leads to meiosis seems also to be transduced via the Ras/cAMP pathway. Mutations that inactivate cAPK (*e.g. cdc35*), result in diploids that induce sporulation even in the presence of nutrients. Mutations that confer high activity of cAPK (*e.g. bcy1*) result in diploids that are deficient for meiosis and sporulation (Shilo *et al.*, 1978; Matsumoto *et al.*, 1983). Control of meiosis by the Ras/cAMP pathway has been implicated to be exerted via the *IME1* gene, an activator of early meiotic gene expression. Bowdish *et al.* (1995) demonstrated that Ime1 acts by binding to the repressor Ume6 which in turn becomes an activator of early meiotic genes (Bowdish *et al.*, 1995). Overexpression of *IME1* enables cells to sporulate in the presence of nutrients, which means that Ime1 is able to override the starvation signal (for a review on meiosis see Mitchell, 1994). It has been suggested that entry into meiosis is regulated through *IME1* by the Ras/cAMP pathway, and accordingly *ime1* has been shown to be epicstatic to *ras2* and *cdc25* (Matsuura *et al.*, 1990). However, until now no direct link has been identified between the two pathways.

During our studies of Rim15 and its role in nutrient signaling we also observed that diploid *rim15Δ* cells were defective for sporulation, which, as described above, is also a phenotypic trait of *bcy1* cells. Most interestingly, while our study was in progress, the *RIM15* gene has been identified through a screen for mutations that causes reduced expression of *IME2*, an early meiotic gene (Vidan and Mitchell, 1997). The authors showed that a diploid *rim15Δ* strain had reduced levels of early meiotic transcripts. Furthermore they were able to demonstrate that diminished Ime1-Ume6 complex formation cosegregated with *rim15Δ*. Therefore, current knowledge suggests that Rim15 may act by stimulating formation of the Ime1-Ume6 complex and thereby expression of early meiotic genes. These results are highly interesting since they indicate that Rim15 could be the missing link between the Ras/cAMP pathway and the induction of early meiotic genes.

Outlook - A glimpse over the *RIM*

The results presented in this thesis have provided answers to a number of questions about trehalose synthesis and its regulation. They have also answered a number of questions that were not asked in the beginning of this project. And what makes science so interesting is that with each answer at least three new questions will arise. Therefore, it is now time to risk a look into the future and to see which of the new questions may be most interesting to try and answer next.

1. The exact nature of the relationship between Rim15 and trehalose synthesis has unfortunately not been fully elucidated. The possibility, discussed above, that Rim15 may act on one of the regulatory subunits, Tsl1 or Tps3, seems worth to follow. Kinase assays with epitope-tagged Tsl1 or Tps3 and GST-Rim15 would be the first step. It would also be possible to analyze the consequences of *RIM15* overexpression in various deletion mutants (e.g. *tps2Δ*, *tps3Δ*, and *tsl1Δ*) of the Tre6P synthase/phosphatase complex during exponential phase. This may indicate which subunit is important for a possible Rim15-dependent regulation.

2. The identification of Rim15 as a downstream effector of cAPK in *S. cerevisiae* is an exciting result, even more since it seems as if Rim15 may also be the link between the Ras/cAMP pathway and meiosis. A next interesting and logical step would be the isolation of downstream targets of Rim15. These may involve factors controlling the cell cycle machinery, trehalose synthesis and elements of the sporulation pathway. Classical genetic screens or two-hybrid screens would be possible ways to proceed.

3. The model presented in the General Discussion also suggests the action of a number of cAMP-independent regulatory mechanisms upon Rim15. It will be interesting to learn more about the nature of these regulatory mechanisms. These may be transcription activators or repressors, as well as activating proteins like protein phosphatases that are expected to relief cAPK-mediated inhibition of Rim15 upon the diauxic shift.

4. A number of other nutrient signaling pathways are involved in cell cycle progression and growth control, in addition to the Ras/cAMP pathway. The possible relationship of Rim15 with these pathways, for instance with the Snf1, the Tor, the Whi2, the Yak1, or the Sch9 pathway has not yet been elucidated. It would be very interesting to find out whether any of these pathways might converge at Rim15.

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Curriculum Vitae

Anke Reinders

April 25, 1968	Born in Bremen, Germany
1974-1978	Primary school in Bremen
1978-1980	Orientierungsstufe in Bremen
1980-1987	Gymnasium in Bremen (closing with Abitur)
1987-1993	Enrolled at the University of Bremen, study of biology, (major: botany, minors: cell and molecular biology, marine biology, biochemistry) Diploma work under the supervision of Prof. Dr. Wolfgang Heyser on "Determination of storage carhohydrates in the mycorrhizal fungus *Suillus bovinus* (Fr.) O. Kuntze"
1990-1991	Exchange student at the University of Maryland, Department of Botany Research project under the supervision of Prof. Dr. Heven Sze on "Reassembly and recovery of the ATPase and proton-transport activity of the vacuolar proton-translocating ATPase of oats"
1994-1998	Ph.D. course at the Botanical Institute of the University of Basel under the supervision of Prof. Dr. Andres Wiemken, Prof. Dr. Thomas Boller, and Dr. Claudio De Virgilio Defense of a Ph.D. thesis with the title "Trehalose synthesis and nutrient signaling in the yeast *Saccharomyces cerevisiae*"

During my studies I visited lectures of the following scientists:

D. Beyersmann, T. Boller, C. De Virgilio, H. Flohr, D. Gabel, L. H. Grimme, W. Heyser, A. Hildebrandt, B. Hohn, T. Hohn, B. Jastorff, G. Kattner, G.-O. Kirst, C. Körner, J. Lockard, F. Meins, D. Mossakowski, J. Motta, J. Müller, A. Nehrkorn, J. Oetiker, N. Page, R. Racusen, L. Rensing, G. Roth, M. Rumpho, G. Schatz, K.Schautz, M. Schirmer, W. Schloot, G. Small, V. Smetacek, A. Sturm, H. Sze, G. Thiel, A. Vallbracht, R. Vögeli-Lange, H. Walter, J. Watson, G. Weidemann, A. Wiemken, H. Witte

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