The Ras/cAMP pathway in yeast: Identification and analysis of new effectors/activators involved in cell proliferation control

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List of Abbreviations

aa	amino acids
ATP	adenosine-5'-triphosphate
bp	base pairs
BSA	bovine serum albumine
cAMP	cyclic adenosine-3',5'-monophosphate
CDC	cell division cycle
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
EDTA	ethylenediaminotetraacetic acid
FGM	fermentable growth medium
G protein	GTP-binding protein
GAP	GTPase-activating protein
GEF	guanine exchange factor
GFP	green fluorescent protein
GPCR	G protein coupled receptor
GST	glutathione-S-transferase
GTP	guanosine-5'-triphosphate
HA	hemagglutinin
HOG	high osmolarity glycerol
Hsp	Heat shock protein
LRR	leucine-rich repeats
MAP	mitogen activated protein
ORF	open reading frame
PCR	polymerase chain reaction
PDS	post-diauxic shift
PKA	cAMP dependent protein kinase A
RGS	regulator of G protein signaling
RNA	ribonucleic acid
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
STRE	stress-responsive element
ts	temperature sensitive

General Introduction

During evolution, life on earth has advanced from simple, unicellular species to complex, multicellular organisms. Cells that live in and build up such a multicellular organism have given up their independence and have become specialized cells that fulfill their function, for instance, as part of a tissue or an organ. A crucial process for the development and the life cycle of cells within these higher structures of cell assemblies is the coordination of growth and metabolism to form a functional unit. In order to achieve this, the cells had to evolve systems that allow extensive cross-talk between each other, and that enable cells to sense signals from both neighboring cells and the overall environment. Cells that loose control over these crucial coordination programs may start to differentiate and grow in an unpredictable way and ultimately even cause the formation of cancer.

Cross-talk between cells is accomplished mainly by the use of chemical signal molecules such as hormones, neurotransmitters or growth factors. These molecules are generally secreted by a signaling cell and then recognized by a target cell where it binds to a plasma membrane receptor. Binding of the signal molecule activates a process called signal-transduction, which involves a cascade of modifications of intracellular proteins or molecules commonly referred to as second messengers. The transduced signal elicits a response which then leads to an adaptation of the cells metabolism to a corresponding new situation. It is not surprising therefore, that many proto-oncogenes that have been identified so far code for mutant signaling molecules. Accordingly, cells that become unresponsive to a proliferation inhibitory signal due to a defective signaling protein may be prone to uncontrolled cell growth and give rise to a population of cancer cells. Therefore, molecular understanding of signal transduction processes is a main research objective that already does and is likely to continue to form an important basis for cancer therapies.

Bakers' yeast *Saccharomyces cerevisiae* has been the designated model system for studying eukaryotic cells for decades now. Its easy yet powerful genetics combined with the nowadays completely sequenced genome make it a valuable, if not indispensable tool to study the biochemical processes inside a single cell. Although this unicellular fungus may lack the inter-cellular communication mechanisms as they are found in cells of multicellular arrangements like organs, the molecular basis of signal transduction pathways can still be studied in this organism. Yeast cells have to deal with changing environmental conditions, including nutrient starvation, temperature stress and osmotic stress. All these events have to

be sensed by the cell so it can respond in an appropriate way to survive and compete in its natural habitat. Importantly, it has been found that the signal transduction mechanisms that are used by yeast cells are highly conserved between eukaryotic organisms. One of the most extensively studied pathways in yeast, for example, is the pheromone response pathway, which is activated when two haploid yeast cells of opposite mating types prepare for mating. Some of the subsequent events following binding of the pheromone to a transmembrane receptor involve the successive phosphorylation of several protein kinases, the last of which is the mitogen activated protein (MAP) kinase homolog. The MAP kinase cascade is a signal transduction module that is used as part of many other signaling pathways present in yeast and higher eukaryotes. Notably, the extracellular signals that activate the MAP kinase may be as diverse as the resulting responses, including mating and pseudohyphal growth in yeast and growth factor-induced differentiation in mammalian systems.

Another well characterized signal transduction mechanism in yeast that finds its homologous equivalent in other eukaryotes is signaling via the intracellular second messenger 3',5'-cyclic AMP (cAMP). An extracellular stimulus can lead to activation of adenylate cyclase, which produces cAMP from ATP and thereby increases the intracellular cAMP concentration. This pathway is of special interest in yeast, since it regulates growth and cell cycle progression in response to nutritional conditions. In the presence of abundant fermentable carbon sources, elevated cAMP levels promote proliferative growth via activated cAMP dependent protein kinase A (PKA). Nutrient limitation on the other hand causes inactivation of the pathway. As a result, cells stop growth, arrest in G_1 and exit the cell cycle into a quiescent state, also known as stationary phase, which is equivalent to the G_0 state of higher eukaryotes.

While in the classical eukaryotic model, adenylate cyclase is under control of a special class of guanine nucleotide binding proteins (G proteins), namely the heterotrimeric G proteins, it was found in yeast that activation of adenylate cyclase is mediated by the small monomeric G proteins Ras. Recently however, a second G protein has been identified that controls adenylate cyclase activity, which shares homology to the G_α subunit of heterotrimeric G proteins. In addition, an associated G protein coupled receptor (GPCR) has also been discovered. Since both proteins have been shown to be involved in glucose-induced cAMP signaling, a model in which the receptor acts as a glucose sensor and signals via the G_α protein brings back the yeast system in line with the classical concept of higher eukaryotes. This renders studies on the PKA pathway even more attractive, because it is likely that the obtained results may be conserved and transferable to other systems.

While the molecular events that lead to activation/deactivation of PKA are well established, little is known on how PKA regulates growth and coordinates progression through the cell cycle. Many cellular processes are known to be regulated by PKA activity, but the targets and molecules that mediate these adaptations remain elusive. One of the known targets of PKA is the Rim15 protein kinase, which has been shown to control a broad set of the nutrient-limitation induced adaptations in a PKA-dependent manner. Accordingly, it is released from negative regulation by PKA upon nutrient starvation and subsequently activates the G_0 program. The molecular basis of this activation, however, remains still unknown.

Given the importance of Rim15 for proper stationary phase entry, this study aims at identifying new targets and effectors of this protein kinase, to gain more insight in how the PKA nutrient signaling pathway coordinates growth and cell cycle progression.

Chapter I: Introduction

Signaling through the cAMP/PKA pathway in *Saccharomyces cerevisiae*

Signaling through the cAMP/PKA pathway in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a facultative anaerobic microorganism. It shows a diauxic growth, which means it can use glucose, sucrose and fructose by fermentation, but also non-fermentable carbon sources like ethanol, lactate or acetate by respiration. The preferentially used sugar is glucose, which is fermented to CO_2 and ethanol even under aerobic conditions. When glucose is depleted, in a process called diauxic-shift, the cells adapt their metabolism to the use of the ethanol that was produced during the fermentative growth.

The level of glucose in the environment must be transmitted rapidly to the cellular machinery to allow appropriate adjustments of the cellular metabolism. The glucose-induced switch from respiration to fermentation is partially mediated by the main glucose repression pathway (Ronne, 1995), which serves to turn off expression of genes whose products are needed for gluconeogenesis. The glucose repression pathway is regulated by the Snf1 protein kinase, which mediates glucose repression largely by negatively controlling the transcription factor Mig1. In parallel to the glucose repression pathway, another nutrient sensing pathway signals through the second messenger cAMP, which activates PKA and thereby triggers a broad range of adaptations in the cell that are necessary for the rapid switch to fermentation. Active PKA reflects the availability of abundant glucose and promotes proliferative growth. Glucose depletion, on the other hand, causes inactivation of the cAMP/PKA pathway and, as a result, causes cells to arrest in G_1 and eventually exit the cell cycle and enter stationary phase. Notably, the cAMP/PKA pathway regulates a broad range of growth control aspects in addition to the control of the carbon-source-dependent metabolic switch. The different signals and stimuli that modulate the activity of the cAMP/PKA pathway, the components used for transmitting the signal (see Fig. 1), and the diverse targets and their effects on the cell and its metabolism are introduced in more detail in the following sections.

The components of the Ras/cAMP pathway

The small monomeric G proteins Ras1 and Ras2

Amongst the superfamily of the G proteins, several classes are important for signaling, like the regulatory GTP hydrolases, which include the monomeric Ras proteins, translation elongation factors and heterotrimeric G proteins. They all share a common structural core, the

guanine nucleotide binding domain (Bourne *et al.*, 1990; Bourne *et al.*, 1991), and their activation state is defined by the bound guanine nucleotide. Accordingly, G proteins associated with GTP are in general active and the intrinsic GTP hydrolase activity promotes the transition to the inactive, GDP bound state. Both states, as well as the transition states, are subjected to regulation by additional accessory proteins, which will be introduced below in more detail.

The group of small GTP-binding proteins is composed of the Ras, Rho, Rab, Scr1/Abt and Ran families. The Ras protein is a membrane-bound protein which harbors just the minimal structural requirements for guanine-nucleotide binding and intrinsic GTPase activity. It was first discovered in 1978/1979 as the transforming agents of Harvey and Kirsten rat sarcoma viruses (Shih *et al.*, 1978; Chien *et al.*, 1979). Subsequently, Ras homologs were found in many eukaryotic systems and described as critical regulators of numerous cellular processes, including cell proliferation (Dent *et al.*, 1992; Huang *et al.*, 1993; Macdonald *et al.*, 1993),

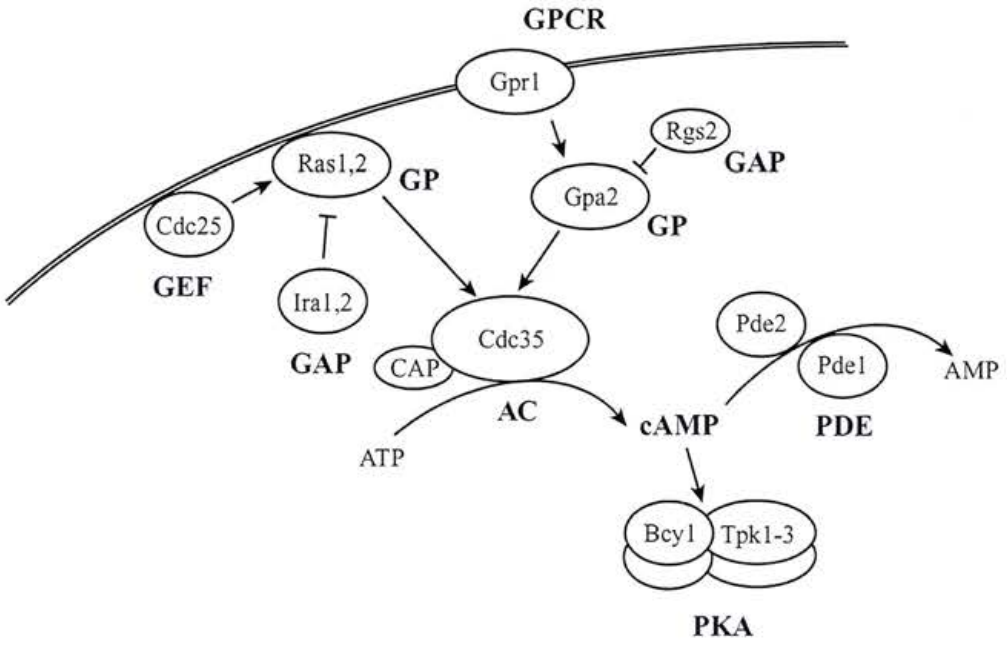

Fig. 1 The components of the Ras/cAMP/PKA pathway. cAMP is synthezised by adenylate cyclase (AC) and degraded by phosphodiesterases (PDE). Adenylate cyclase is activated by two G proteins (GP), that are themselves subject to regulation by inhibitory GTPase-activating proteins (GAP) and an activatory GDP/GTP exchange factor (GEF) for the Ras proteins or an activatory G protein coupled receptor (GPCR) for Gpa2. Notably, protein kinase A (PKA) is a holoenzyme of two regulatory (Bcy1) and two catalytic (Tpk1-3) subunits. PKA is activated by binding of cAMP to the regulatory subunits followed by their dissociation from the catalytic subunits.

differentiation (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Hagag *et al.*, 1986), morphogenesis (Feramisco *et al.*, 1984; Bar-Sagi and Feramisco, 1986) and apoptosis (Kauffmann-Zeh *et al.*, 1997) in mammals, as well as insulin-induced maturation of oocytes in amphibians (Korn *et al.*, 1987), photoreceptor development in fruit flies (Dickson and Hafen, 1993) and vulval cell development in nematodes (Han and Sternberg, 1990). Importantly, activated Ras oncogene products have been found to cause of a wide variety of tumors (30% of human tumors).

In mammals, there exist the three nearly identical Ras proteins, namely H-Ras, N-Ras and K-Ras, which differ mostly in their carboxy-terminal parts (Taparowsky *et al.*, 1983). They are activated by receptors with intrinsic or associated tyrosine kinase activity that respond to various extracellular signals. Interestingly, yeast cells lack receptor tyrosine kinases and the activatory mechanisms of Ras via putative receptor proteins remains elusive.

The two Ras proteins found in yeast, Ras1 and Ras2, share a high degree of homology with mammalian Ras proteins in their N-terminal domains (DeFeo-Jones *et al.*, 1983; Powers *et al.*, 1984; de-Araujo, 1996). The two proteins are 322 and 309 amino acids in size, respectively, and thus considerably larger than their closest homologue, the 189 amino acid H-Ras. Accordingly, both proteins have an extended, variable C-terminus that shares no homology with H-Ras. Ras1 and Ras2 are not essential by themselves, but loss of both proteins causes growth arrest. Growth can be restored to *ras1ras2* double mutants by expression of mammalian H-Ras (Kataoka *et al.*, 1985b). In addition, expression of an activated yeast $RAS2^{Val19}$ allele in mammalian cells induces proliferative transformation in a mammalian cell line (DeFeo-Jones *et al.*, 1985). Thus, yeast and mammalian Ras proteins are functionally interchangeable. Yeast *RAS1* and *RAS2* are differentially expressed depending on nutrient availability, which indicates that even though the proteins functionally overlap, they may also have separate functions (Breviario *et al.*, 1988).

Ras proteins are post-translationally modified and it could be shown, that this modification is essential for their subcellular localization as well as for their biological function. The modifications are essentially the same for mammalian and yeast Ras proteins (Fujiyama *et al.*, 1987; Deschenes *et al.*, 1989; Finegold *et al.*, 1990; Fujiyama and Tamanoi, 1990; Goodman *et al.*, 1990). They include three sequential modifications of a CAAX tetrapeptide, which is conserved in all Ras proteins (Casey, 1994). In yeast, the Ras proteins are farnesylated at the cysteine of this motif by a farnesyltransferase, a heterodimeric protein complex encoded by *RAM1* and *RAM2*. This step is required for all subsequent modifications. After proteolytic

removal of the terminal three amino acids by the proteases Acf1 and Rce1, the farnesyl residue is carboxymethylated by the carboxymethyl transferase Ste14 (Clarke *et al.*, 1988; Fujiyama and Tamanoi, 1990; Schafer and Rine, 1992). Based on the finding that mutations in *RAM1* and *RAM2* were isolated as extragenic suppressors of an activated $RAS2^{Val19}$ allele (Powers *et al.*, 1986; He *et al.*, 1991), it was suggested that farnesylation of Ras proteins is not only important for localization, but also for their biological function. In accordance with this suggestion, it has been shown that farnesylation of Ras proteins is indeed essential to enhance their interaction with their downstream target adenylate cyclase (Kuroda *et al.*, 1993; Shima *et al.*, 1997). Moreover, it was found that farnesylation is also required for stimulation of the GDP to GTP exchange by the full-length guanine nucleotide exchange factor Cdc25 (Crechet *et al.*, 2000, and see below). In the mammalian system, the corresponding modifications were also reported to enhance the specific interaction of Ras proteins with their target proteins such as Raf, PI3-K or MEKK1 (Takai *et al.*, 1992). In this context, it could recently be shown that the dominant activate $p21^{ras}$ allele in mammals loses its transforming activity if it is not farnesylated (Rowinsky *et al.*, 1999; Scharovsky *et al.*, 2000). For this reason, farnesyltransferases have now become important targets in anticancer therapeutic research.

Since the main function of the Ras protein farnesylation is apparently not in membrane targeting, it seems that Ras farnesylation may also not be sufficient for stably anchoring of Ras proteins to the membrane. Indeed, additional palmitoylation of one or two cysteine residues immediately upstream of the CAAX box is necessary for membrane localization of Ras (Bhattacharya *et al.*, 1995). In contrast to farnesylation, palmitoylation seems not to have any function with respect to Ras activity or maturation, it is strictly needed to properly localize Ras to the membrane. Notably, this function is essential for successful glucose-induced signaling, indicating that interaction of Ras with its effectors occurs at the cell membrane.

Given the importance of the Ras proteins for normal growth and development of cells and the dramatic effects of the oncogenic alleles in mammals, identification of the elicitors and the downstream effectors of these proteins is of increasing interest. In *Saccharomyces cerevisiae*, studies involving Ras and cAMP metabolism mutants first indicated that the Ras proteins may regulate the activity of adenylate cyclase (Broek *et al.*, 1985; Toda *et al.*, 1985). Accordingly, $RAS2^{Val19}$ mutants and strains with elevated, unregulated cAMP levels were shown to be defective in undergoing a proper diauxic transition and in arrest at the START point of the

cell cycle upon nutrient limitation during growth in a batch culture (Pringle and Hartwell, 1981; Toda *et al.*, 1985; Sass *et al.*, 1986). Moreover, temperature sensitive mutants in Ras or adenylate cyclase were found to arrest at G_1 when shifted to the restrictive temperature (Matsumoto *et al.*, 1985; De Vendittis *et al.*, 1986). Thus, nutrient-limitation induced processes such as entry into the diauxic phase and proper G_1 arrest have been suggested to be conferred by a mechanism involving Ras and cAMP levels (Mbonyi *et al.*, 1988; Dumont *et al.*, 1989; Gibbs and Marshall, 1989; Broach, 1991). Indeed, more recent results not only showed that fermentable sugars activate Ras, but also that Ras directly activates adenylate cyclase (Shima *et al.*, 1997).

The GDP/GTP exchange factors Cdc25 and Sdc25

Like other G proteins, the Ras proteins are active in the GTP-bound form and inactive in the GDP-bound form (Field *et al.*, 1987). Some of the known *ras* oncogenes code for Ras variants which are trapped in GTP-bound, activated form. Interestingly, corresponding yeast Ras2 variants have been created, with the help of which the nature of the activatory mechanism could be studied. $Ras2^{Val19}$ for example was found to display greatly reduced GTPase activity associated with an increased stability of the GTP-bound state, leaving Ras2 predominantly in an activated state (Kataoka *et al.*, 1984; Toda *et al.*, 1985). Consequently, strains expressing such an allele show all characteristics of strains with elevated cAMP levels (Thevelein, 1994). Similar effects were caused by a $RAS2^{Ile152}$ mutation which increases the intrinsic GTPase activity, strongly destabilizes of the GDP-bound state and enhances GDP to GTP exchange (Crechet *et al.*, 1990a). Since the low intrinsic exchange activity of the Ras proteins retains Ras proteins in the GDP bound state under physiological conditions (Proud, 1986), it was suggested that there may be additional mechanisms that control the transitions between the GTP- and GDP-bound states.

Proteins that can catalyze the exchange reaction of nucleotides are common in nature and are generally named GDP/GTP-exchange factors (GEFs). The attempts to identify a GEF for the Ras proteins led to the discovery of Cdc25. The *CDC25* gene was isolated by complementation of the temperature-sensitive growth defect of the cell division cycle mutant *cdc25* (Broek *et al.*, 1987). Strains with deleted reduced levels of Cdc25 function have low cAMP levels and decreased adenylate cyclase activity (Camonis *et al.*, 1986; Broek *et al.*, 1987; Marshall *et al.*, 1987). Thus, such strains show characteristics of strains with an inactive

Ras/cAMP pathway. Importantly, the accompanying phenotypes could be suppressed by overexpression of the downstream effectors of Ras (*e.g.* the adenylate cyclase Cdc35), by introduction of a dominant active $RAS2^{Val19}$ allele, or by overexpression of the downstream acting PKA (Tpk1), indicating that Cdc25 may affect the activation state of the Ras protein. Subsequently, it was indeed demonstrated that the Cdc25 protein catalyses the exchange of guanine nucleotides bound to Ras *in vitro* (Jones *et al.*, 1991). Since deletion of *CDC25* is lethal, Cdc25 was suggested to be required for the basal activity of the protein in addition to its function as an activator of Ras by enhancing the GDP to GTP exchange (Thevelein, 1992). Up to date, Cdc25 homologs have been identified in other eukaryotic organisms (*e.g.,* Ste6 in *Schizosaccharomyces pombe*, SOS in *Drosophila melanogaster* and p140 Ras GRF in mammals) where they were shown to function as activators of Ras proteins (Bonfini *et al.*, 1992). A homolog of Cdc25, namely Sdc25, has also been identified in yeast. The C-terminal domain of Sdc25 catalyses the exchange of guanine nucleotides bound to yeast and mammalian Ras *in vitro* (Crechet *et al.*, 1990b; Boy-Marcotte *et al.*, 1996). Although this truncated variant can suppress a *cdc25* null mutation *in vivo* (Broek *et al.*, 1987; Damak *et al.*, 1991; Boy-Marcotte *et al.*, 1996), its cellular role remains still unclear, since full-length Sdc25 cannot suppress the lethality of a *cdc25* mutant (Damak *et al.*, 1991), nor do *sdc25* mutants show any apparent phenotype.

The Cdc25 protein is located at the plasma membrane, a process which involves hydrophobic sequences at the C-terminal domain (Damak *et al.*, 1991; Garreau *et al.*, 1996). The N-terminal domain, in contrast, is involved in regulation of the interaction between Cdc25 and farnesylated Ras (Crechet *et al.*, 2000). Studies of Cdc25 aiming at elucidating the mechanism by which the GDP to GTP exchange occurs revealed that Cdc25, via association with Ras, forms a nucleotide free complex (Lai *et al.*, 1993; Jacquet *et al.*, 1995). This complex can be dissociated by addition of either GTP or GDP, suggesting that Cdc25 generally catalyses the exchange reaction in both directions, depending on the intracellular GTP and GDP levels (Haney and Broach, 1994; Lenzen *et al.*, 1998; Rudoni *et al.*, 2001). Since GTP is manifold more abundant than GDP inside the cell, Cdc25 is acting mostly as an activator of Ras. In accordance with these findings, the cytosolic GTP/GDP ratio is closely correlated with the relative amount of GTP-bound Ras *in vitro* and *in vivo* (Proud, 1986).

It is likely that Cdc25 is regulated via posttranscriptional mechanisms that include phosphorylation of the protein through cAMP dependent protein kinase. This process may cause relocalisation of Cdc25 to the cytoplasm and dissociation from membrane-bound Ras,

as well as subsequent degradation involving the N-terminal cyclin destruction box that targets proteins for ubiquitin-dependent proteolysis (Kaplon and Jacquet, 1995; Chen *et al.*, 2000).

The GTPase-activating proteins Ira1, Ira2

The transition from the active GTP-bound to the inactive GDP-bound state of the Ras proteins is also subject to regulation. In yeast, two proteins coding for Ras GTPase-activating proteins (GAPs) have been found, namely Ira1 and Ira2 (Inhibitor of Ras) (Tanaka *et al.*, 1989; Tanaka *et al.*, 1990b). When either one of Ira1 or Ira2 is deleted, mutant cells show the typical phenotypes of activated Ras proteins (such as Ras^{Val19}), including elevated cAMP levels. Absence of both Ira proteins leads to accumulation of Ras-GTP because of the absence of an activating mechanism of the Ras-intrinsic GTPase activity. So in yeast, the Ira proteins function analogously to mammalian GAPs. They negatively regulate Ras proteins by stimulating their intrinsic GTPase activity.

The homology to the mammalian GAPs extends only over the 330 amino acids of the catalytic core domain of the Ira proteins. While the catalytic domain of human GAP Neurofibramin NF-1 can substitute for the Ira proteins in yeast (Ballester *et al.*, 1989; Tanaka *et al.*, 1990b), the yeast proteins seem not to act on mammalian Ras proteins (Adari *et al.*, 1988; Tanaka *et al.*, 1990a; Tatchell, 1993; Parrini *et al.*, 1995), indicating that the remaining parts of the Ira protein may define specificity towards yeast Ras proteins.

The adenylate cyclase

Adenylate cyclase in yeast is encoded by the *CYR1/CDC35* gene (Matsumoto *et al.*, 1982; Casperson *et al.*, 1983; Matsumoto *et al.*, 1984). It shares homology to the mammalian adenylate cyclase only in its catalytic domain. The other domains of the large yeast adenylate cyclase show no significant similarities to the mammalian counterpart (Kataoka *et al.*, 1985a). This structural difference is accompanied by a difference in subcellular localization, i.e. adenylate cyclase in mammals is an integral membrane protein, whereas the yeast adenylate cyclase is only peripherally associated with the plasma membrane (Mitts *et al.*, 1990).

As mentioned above, the adenylate cyclase in yeast is activated by Ras proteins. In mammals, in contrast, adenylate cyclase is regulated by stimulatory and inhibitory G proteins (G_S and G_I

respectively), that are part of heterotrimeric G protein complexes (Toda *et al.*, 1985). It is likely, therefore, that the additional domains in the yeast adenylate cyclase are involved in receiving and/or transmitting signals from the Ras proteins. Closer analysis of the N-terminal part of Cdc35 showed that it has an inhibitory effect on the catalytic activity of the protein. Accordingly, cleaving off the N-terminal 480 amino acids significantly increased adenylate cyclase activity, suggesting that active Ras may induce cAMP synthesis by releasing this inhibition (Heideman *et al.*, 1990; Uno *et al.*, 1990).

In the central domain ranging from aa 657 to aa 1301, so called leucine-rich repeats (LLRs) have been identified. This domain consists of 26 repeats of an approximately 23 amino acid stretch containing a high amount of leucine and/or other aliphatic residues (Kobe and Deisenhofer, 1994). LLRs are known in several systems to mediate protein-protein interaction and are required for activation of adenylate cyclase by Ras (Colicelli *et al.*, 1990; Field *et al.*, 1990).

The catalytic domain positioned at aa 1667 to aa 1891 has a high homology to adenylate cyclases of other organisms (Yamawaki-Kataoka *et al.*, 1989). It catalyses the formation of cAMP from ATP. In yeast, there is evidence that this domain is also subjected to regulation by the Ras proteins. A mutation in the catalytic domain at position 1876 named *lcr1*, results in an impaired glucose-induced cAMP signal response, while the basal cAMP levels are not affected by this mutation (Vanhalewyn *et al.*, 1999).

The C-terminus of Cdc35 contains a Ras-binding site (Suzuki *et al.*, 1990; Shima *et al.*, 2000). Furthermore, a protein named CAP (cyclase associated protein) has been identified, whose N-terminal part binds to the C-terminus of Cdc35. Cap seems also to play a role in Ras-dependent regulation of cyclase activity, since loss of Cap reduces the stimulatory effect of mature Ras on the catalytic activity of adenylate cyclase (Gerst *et al.*, 1991; Wang *et al.*, 1992; Wang *et al.*, 1993; Shima *et al.*, 2000). Cap has also been shown to have a Ras-independent function, which is associated with regulation of cytoskeleton formation (Gieselmann and Mann, 1992; Wang *et al.*, 1993; Freeman *et al.*, 1995).

Finally, the region between the LLR and the catalytic domain (spacer region) is also involved in regulation of adenylate cyclase through Ras. A truncated protein consisting only of the catalytic domain and the C-terminus is unresponsive to environmental changes (Uno *et al.*, 1987), whereas addition of the adjacent spacer region to this protein restores its Ras-responsiveness. These results are further supported by identification of a threonine 1651

residue in this region, which changes Ras-responsiveness when replaced by other amino acids (Feger *et al.*, 1991).

The phosphodiesterases Pde1 and Pde2

cAMP levels in the cell are not only regulated by adenylate cyclase-dependent synthesis, but also by phosphodiesterase-mediated degradation. Two phosphodiesterases are found in yeast that catalyze the degradation of cAMP to AMP, namely Pde1 and Pde2. Pde2 is a high affinity phosphodiesterase, orthologs of which are found in many other species (Suoranta and Londesborough, 1984; Charbonneau *et al.*, 1986). The mammalian proteins are known to be regulated by phosphorylation and some of them are involved in control of agonist-induced cAMP responses (Conti *et al.*, 1995). Whereas the K_m of the yeast phosphodiesterase Pde2 lies in the range of the estimated intracellular cAMP level (Suoranta and Londesborough, 1984), Pde1 has a K_m of 5 mM and represents a low affinity phosphodiesterase (Fujimoto *et al.*, 1974; Londesborough and Lukkari, 1980). Pde1 is important for downregulation of high cAMP levels following short-time induction, a process which is controlled by PKA-mediated phosphorylation (Ma *et al.*, 1999). In contrast, Pde2 seems more to be involved in control of the basal cAMP level during growth. Interestingly, deletion of both *PDE* genes was found to suppress deletion of *RAS2*. Since this triple *pde1 pde2 ras2* strain shows also all phenotypes associated with elevated levels of cAMP, unregulated adenylate cyclase is able to produce an excess of cAMP (Nikawa *et al.*, 1987b).

Several mutations in *PDE2* were found to allow cells to take up external cAMP, indicating that Pde2 may protect cells from high external cAMP levels (Mitsuzawa, 1993; Wilson *et al.*, 1993).

The G_α protein Gpa2

As mentioned above, unlike in yeast, mammalian adenylate cyclase is not regulated by monomeric G proteins such as Ras, but rather by heterotrimeric G proteins (Gilman, 1984). Heterotrimeric G proteins consist of three different subunits (*e.g.* G_α, G_β and G_γ). The most diverse in structure and function, as well as the largest of these three is the G_α subunit, which contains the guanine-nucleotide binding site.

The GDP-bound form of G_α is in general inactive and tightly associated with the $G_{\beta\gamma}$ complex. Activation, accompanied by transformation into the GTP-bound form causes dissociation of G_α from the $G_{\beta\gamma}$ subunits. Activation by GDP to GTP exchange is usually induced by activated cell-surface transmembrane receptors, called G protein-coupled receptors (GPCRs). Upon dissociation of G_α from $G_{\beta\gamma}$ subunits, both components may regulate downstream targets depending on the signaling system (Birnbaumer, 1992). Stimulating G_α subunits ($G_{\alpha s}$) activate adenylate cyclases (Tang and Gilman, 1992), as well as Ca^+ and Na^+ channels (Mattera *et al.*, 1989; Schubert *et al.*, 1989). Inhibitory G_α subunits ($G_{\alpha i}$), in contrast, inhibit adenylate cyclase and are involved in regulation of ion channels and cGMP phosphodiesterases (Fung *et al.*, 1981; Birnbaumer *et al.*, 1990). There are also additional members of the G_α class of proteins, namely $G_{\alpha q}$, which can activate phospholipase C (Smrcka *et al.*, 1991). The $G_{\beta\gamma}$ subunit on the other hand can propagate a signal by activating PLC-β2 (Camps *et al.*, 1992; Katz *et al.*, 1992), phosphoinositide-3-kinase (Stephens *et al.*, 1994) and even specific isotypes of adenylate cyclase (Tang and Gilman, 1992). In yeast, the $G_{\beta\gamma}$ subunit Ste8/Ste14 acts in the mating pathway by activating protein kinase Ste20 (Abate *et al.*, 1990; Nomoto *et al.*, 1990; Akada *et al.*, 1996).

Similar to the small monomeric G proteins, maturation of heterotrimeric G-protein subunits by post-translational lipid modification is essential for their function (Wedegaertner *et al.*, 1995). G_α subunits are in general myristoylated at the N-terminal glycine residue or palmitoylated at an internal cysteine. G_γ subunits contain the CAAX motif and are, similar to Ras, prenylated at the cysteine residue, following which the three C-terminal amino acids are proteolytically removed and the new C-terminus is carboxymethylated. Modifications of G_β subunits have not been described yet.

In yeast, only two genes encoding G_α subunits have been identified, *GPA1* and *GPA2* (Miyajima *et al.*, 1987; Nakafuku *et al.*, 1988). The role of Gpa1 in the mating pathway is well established. The function of Gpa2 remained unclear for several years, but recent results demonstrate that Gpa2 may act as a G_α subunit activating adenylate cyclase, which would bring G protein signaling in yeast back in line with the system found in higher eukaryotes. (At the same time, this questiones the role of Ras1/Ras2 in nutrient signaling in yeast.) In accordance with this, cells overexpressing *GPA2* were found to have elevated cAMP levels and to suppress a temperature-sensitive Ras2 mutation, and cells expressing the constitutively active $GPA2^{Ala273}$ allele were found to have phenotypes that are associated with elevated cAMP levels (Xue *et al.*, 1998). Moreover, the severe growth defect of a *gpa2Δ ras2Δ* mutant

could be suppressed by deletion of *PDE2* (Kubler *et al.*, 1997), indicating a lack of cAMP in strains devoid of Ras2 and Gpa2. Since *GPA2* disruption alone only partially reduced the glucose-induced cAMP formation (Nakafuku *et al.*, 1988), Ras2 and Gpa2 may, however, have some redundant functions in positive regulation of cAMP levels. To dissect the different roles of Ras2 and Gpa2 in cAMP signaling, the different mechanisms by which the Ras/cAMP pathway can be activated have to be studied separately. After pre-incubation in medium containing a low level of glucose, the induction of cAMP synthesis upon addition of high levels of glucose was found to be dependent on Gpa2 (Colombo *et al.*, 1998). On the other hand, cAMP induction after intracellular acidification, triggered by the addition of DNP following pre-incubation on medium containing low-levels of glucose, was not dependent on Gpa2. This led to the conclusion that Gpa2 is essential for high glucose induced cAMP signaling, but not for induction of cAMP synthesis after intracellular acidification.

The obvious attempts to identify the $G_{\beta\gamma}$ subunit that binds to Gpa2 failed so far. Neither deletion of different putative G_{β} and G_{γ} subunits, based on sequence homology, nor the analysis of already known yeast $G_{\beta\gamma}$ subunits influenced cAMP-dependent signaling (Papasavvas *et al.*, 1992; Lorenz and Heitman, 1997).

G_{α} subunits of heterotrimeric G proteins that contain the GTP hydrolysis catalytic domain are, similar to the small monomeric Ras proteins, also subject to regulation by GTPase activating proteins, called Rgs proteins (regulators of G protein signaling, *e.g.* Sst2 in the case of the yeast pheromone pathway) (Dohlman and Thorner, 1997; Iyengar, 1997).

A protein containing a putative RGS domain that may act on Gpa2 was isolated recently and named Rgs2 (Versele *et al.*, 1999). Deletion of *RGS2* caused enhanced glucose-induced accumulation of cAMP, whereas overexpression caused the inverse phenotype. In addition, Rgs2 interacted specifically with the transition state during GTP to GDP hydrolysis of Gpa2 (Versele *et al.*, 1999), which can be mimicked *in vitro* by incubation of the G protein subunit with GDP and AlF_4^- (Hamm and Gilchrist, 1996). Moreover, Rgs2 accelerated the GTP hydrolysis on Gpa2 *in vitro*, supporting a role for Rgs2 as a GAP for the G_{α} subunit Gpa2.

The G protein coupled receptor Gpr1

A putative G protein coupled receptor for Gpa2, as it is found for many other heterotrimeric G proteins, was found in a yeast two-hybrid screen using Gpa2 as a bait (Fields and Song, 1989;

Yun *et al.*, 1997; Xue *et al.*, 1998; Kraakman *et al.*, 1999). This protein, namely Gpr1, shows sequence and structure homology to other GPCRs, such as the seven transmembrane helices, extended N- and C-terminal domains and a large third intracellular loop (Dohlman *et al.*, 1991; Strader *et al.*, 1994). Two observations confirm that Gpr1 could indeed be the activating receptor for the G_α subunit Gpa2. First a *gpr1Δ ras2Δ* double mutant shows a similar growth defect as a *gpa2Δ ras2Δ* double mutant, and both double mutants can be suppressed by deletion of *PDE2* or by addition of extracellular cAMP (Kubler *et al.*, 1997; Xue *et al.*, 1998). These results indicate that Gpa2 and Gpr1 may transmit a signal in the same pathway, possibly in parallel to Ras, and upstream of adenylate cyclase.

Second, a *fil2* mutation (fermentation-induced loss of stress resistance), isolated in a screen for mutants that, upon addition of glucose, are defective in exit from their stage of high stress resistance acquired during growth on a poor carbon source (Kraakman *et al.*, 1999; Thevelein and de Winde, 1999), mapped to a mutation in the large intracellular loop of Gpr1. Strains expressing this Gpr1 mutant protein lack glucose-induced cAMP signaling like a *gpr1Δ* or *gpa2Δ* deletion mutant.

Recent research focused on the elucidation of the ligand of the GPCR Gpr1, which may be glucose itself, given the fact that glucose is responsible for induction of cAMP synthesis. As for other GPCRs, Gpr1 is localized in the membrane and membrane-proximal regions in its cytoplasmic third loop have been shown to be required for proper functioning (Baldwin, 1994; Yun *et al.*, 1997; Xue *et al.*, 1998). Furthermore, it could be shown that in a strain lacking detectable glucose uptake (the *hxt* null strain, see Chapter I: 'Glucose signaling'), cAMP synthesis can still be induced by addition of high concentrations of glucose, but this induction is strictly dependent on the presence of Gpr1 (Rolland *et al.*, 2000). Thus, Gpr1 may well prove to be the receptor for glucose, and internalization of the sugar followed by changes in intracellular glucose concentrations, may not be required for stimulation of cAMP synthesis.

The apparent affinity for glucose would, accordingly, be very low when compared to the high affinity that other GPCR systems exhibit for their ligands. Nevertheless, this is in correspondence with the previous finding, that Gpr1/Gpa2 signaling is required for a response to high concentrations of sugar, but not for regulation of basal levels of cAMP, nor for a response to low levels of glucose. It is also in agreement with the physiological context in which this nutrient sensing system has to function (Thevelein, 1991).

Taken together, there is pretty strong evidence that Gpr1 and Gpa2 in yeast indeed serve as the GPCR/G protein system involved in glucose sensing, yet the direct physical interaction by Gpr1 and its putative ligand glucose remains to be demonstrated.

The cAMP-dependent protein kinase A: PKA

Regulation of cAMP levels in the cell control the activity of PKA, which regulates a multitude of proteins and thereby allows appropriate cellular adaptations. As in higher eukaryotes, the PKA holoenzyme is a tetramer consisting of two regulatory subunits and two catalytic subunits. The catalytic subunits are encoded by the three genes *TPK1*, *TPK2* and *TPK3*. The gene products exhibit redundant functions with overlapping substrate recognition, but have also different activities with respect to specific targets. The regulatory subunit is encoded by *BCY1* (Toda *et al.*, 1987). When the two regulatory subunits are tightly associated to the catalytic subunits, PKA is inactive. PKA is activated by binding of two cAMP molecules to each regulatory subunit, causing their dissociation from the catalytic subunits (Johnson *et al.*, 1987; Toda *et al.*, 1987).

It is generally assumed that subcellular localization of the subunits during distinct physiological states plays an important role in regulating PKA activity. In mammals, the regulatory subunits are almost exclusively found in the cytoplasm, where regulation by cAMP takes place. Upon stimulation, the released catalytic subunits shuttle to the nucleus (Nigg *et al.*, 1985; Meinkoth *et al.*, 1990; Harootunian *et al.*, 1993). In yeast, the situation is clearly different and somewhat more complex. In cells grown on a fermentable carbon source, both subunits, Bcy1 and Tpk1 were predominantly found in the nucleus (Griffioen *et al.*, 2000). The nuclear localization of Tpk1 under these conditions was dependent on the presence of Bcy1, and activation by addition of cAMP led to release of Tpk1 into the cytoplasm. In cells grown on a non-fermentable carbon source or in stationary phase cells, where levels of cAMP are generally low, Bcy1 was found partly in the cytoplasm and the catalytic subunit was found to be excluded from the nucleus. This suggests that Bcy1 regulates subcellular localization of Tpk1. The mechanisms of this regulatory control remains unclear.

The cellular responses regulated by PKA include stress resistance (as a result of control of carbohydrate levels and stress gene expression), glycolysis and gluconeogenesis, ribosomal gene expression, cell growth, and progression through the cell cycle. In the following

chapters, the different PKA targets as well as the different conditions leading to activation of PKA will be discussed in more detail.

Activation of protein kinase A

The different genetic experiments that allowed identification and characterization of the known components of the Ras/cAMP pathway as well as the recent discovery of the Gpr1/Gpa2 system, suggest the presence of different mechanisms by which the nutritional status in general and the presence of glucose in particular is sensed and the signal is transmitted to activate PKA. Current knowledge on the different mechanisms and on their physiological implications, which has changed quite a bit during the last years, is reviewed below (see also Fig. 2).

Glucose sensing

Early studies aiming to identify the means by which glucose triggers the transient cAMP rise and the subsequent activation of PKA, investigated the role of glucose uptake and its metabolism in the cell. As mentioned before, glucose-induced cAMP signaling requires

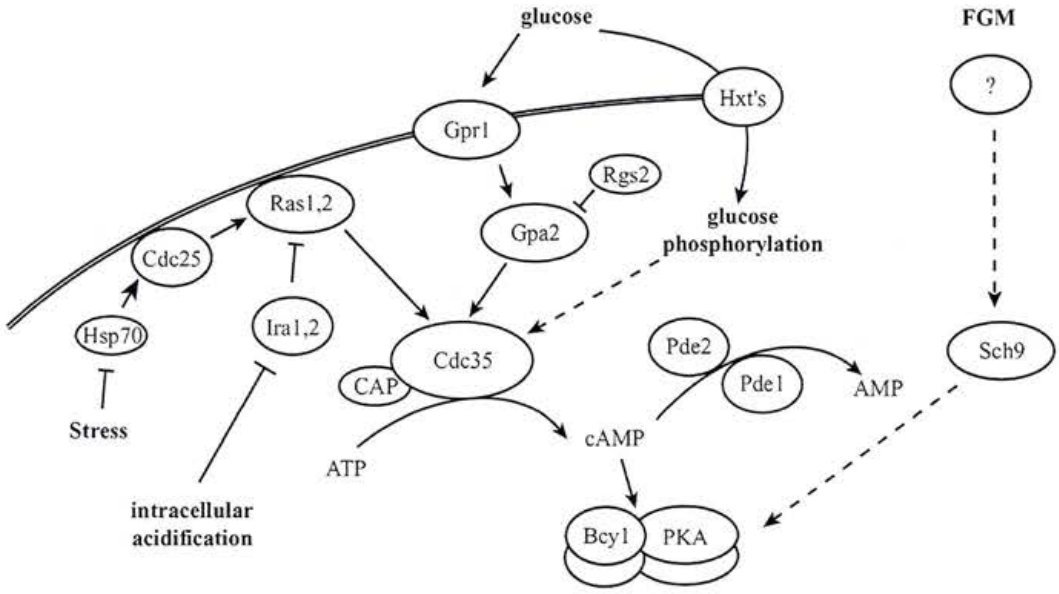

Fig. 2 Conditions leading to activation/deactivation of PKA. For details see legend of Fig.1.

extracellular glucose detection by the Gpr1/Gpa2 G protein coupled receptor system. In addition to Gpr1 and Gpa2, however, glucose activation of cAMP synthesis also requires uptake and phosphorylation of the sugar (Beullens *et al.*, 1988; Pernambuco *et al.*, 1996).

Several glucose carriers have been identified in yeast that facilitate the diffusion of glucose through the membrane (Bisson *et al.*, 1993) and they were classified as hexose transporter genes, *HXT* (Andre, 1995; Kruckeberg, 1996; Boles and Hollenberg, 1997). Of the 20 known *HXT* genes, only seven, (*i.e. HXT1* to *HXT7*) are assumed to be involved in glucose transport. Accordingly, a strain deleted for all those seven *HXT* genes, the *hxt*-null strain, does not show detectable glucose uptake any more. This strain lacks glucose-induced cAMP signaling and can not grow on glucose. This strain, however, grows on galactose, provided that the galactose transporter Gal2 is overexpressed (Reifenberger *et al.*, 1997) or on maltose, provided that the maltose permease Mal1 is overexpressed (Vanoni *et al.*, 1989). Under such conditions, intracellular glucose levels as well as glucose-induced cAMP signaling is restored. This finding suggests that none of the active hexose transporters have a regulatory function in activation of the Ras/adenylate cyclase pathway.

For two *HXT* gene products, which do not actively transport glucose, namely Snf3 and Rgt2, a role in regulating the expression of other *HXT* genes has been demonstrated (Ozcan *et al.*, 1996; Ozcan *et al.*, 1998). It was suggested that they both generate an intracellular glucose signal, which is required for *HXT* expression. Snf3 is required for expression of the *HXT* genes in respect to low levels of glucose and Rgt2 is required for maximal induction of gene expression in response to high levels of glucose. Notably, however, both proteins are not required for glucose-induced cAMP signaling.

The results presented above suggest that glucose phosphorylation, or further metabolism of glucose in the cells are required for proper signaling through adenylate cyclase. In this context, there are three kinases that phosphorylate glucose, i.e. glucokinase Glk1 and the hexokinases Hxk1 and Hxk2 (Lobo and Maitra, 1977), and loss of all these proteins prevents glucose-induced cAMP signaling.

Since thereby glucose-6-P levels nor further metabolism of glucose-6-P is required for induction of cAMP synthesis (Beullens *et al.*, 1988; Rolland *et al.*, 2001), it may be that the sugar kinases themselves have a direct regulatory function. Yet the mechanism by which this is achieved remains unclear.

Intracellular acidification

Two stimuli are known to activate the Ras/adenylate cyclase pathway *in vivo*. As shown above, glucose, or a related rapidly fermentable sugar, triggers activation of the pathway when added to starved cells (van der Plaat, 1974; Purwin *et al.*, 1982; Tortora *et al.*, 1982; Thevelein, 1984a). In addition, conditions leading to intracellular acidification, like for instance addition of the protonophore 2,4-dinitrophenol (DNP) at low extracellular pH (Trevillyan and Pall, 1979), can also activate the pathway. It was initially thought that acidification caused by glucose addition triggers activation of the Ras/cAMP pathway. This assumption was based on the observation that addition of glucose to derepressed yeast cells induces a transient drop in intracellular pH as a result of proton production during the early metabolic steps of glycolysis (Busa and Nuccitelli, 1984; Caspani *et al.*, 1985; Purwin *et al.*, 1986). However, prevention of this pH drop, by addition of NH_4Cl, did not abolish glucose-induced signaling (Thevelein *et al.*, 1987). Recent work shows that glucose- and intracellular acidification-induced stimulation of adenylate cyclase act through different G proteins. As described, glucose-induced cAMP synthesis is dependent on the extracellular glucose sensing process, which is mediated by the GPCR Gpr1 and the G protein Gpa2, whereas intracellular acidification-induced cAMP synthesis is dependent on the Ras proteins, and may involve inhibition of Ras by the GAP proteins Ira1 and Ira2. Accordingly, the rapid increase of GTP on the Ras proteins after intracellular acidification was absent in *ira1Δ ira2Δ* mutants, suggesting that it might be mediated by inhibition of the Ira proteins (Colombo *et al.*, 1998). However, the physiological role of the intracellular acidification effect on cAMP synthesis remains unclear.

Stress conditions

Besides the two stimuli mentioned above, the Ras/cAMP pathway is also known to respond to stress conditions such as heat or osmotic stresses. The perception of these stress conditions remains largely unclear. It has been suggested that the function of the Ras proteins might be controlled by stress-induced heat-shock proteins (Geymonat *et al.*, 1998). Accordingly, it was suggested that the cytosolic Hsp70 Ssa1 directly interacts with and thereby stabilizes Cdc25. Exposure of cells to stress conditions may result in recruitment of Hsps to stress-denatured or

damaged proteins. As a result, Hsps may be drained off Cdc25 which may lead to destabilization of Cdc25 and reduced signaling through the cAMP/PKA pathway.

cAMP independent activation of PKA: the FGM pathway

As mentioned above, PKA is activated upon intracellular acidification and/or by application of glucose to derepressed cells, indicating that the Ras/cAMP pathway adjusts cAMP levels in response to nutrient availability and thereby controls the metabolic activities of the cell. Recent work, however, may change the view on adenylate cyclase and PKA regulation. Measurements of the cAMP levels showed that the differences in cAMP levels between cells grown in rich medium versus stationary phase cells are quite modest. A spike in cAMP levels is usually only observed after the addition of glucose to derepressed cells. Subsequently, cAMP concentrations decrease during exponential growth to levels that do not significantly differ from levels at the diauxic transition, when glucose is depleted (Ma *et al.*, 1997). Importantly, PKA activity is not correlated with this drop in cAMP, and the phenotype of high PKA activity such as low trehalose levels, downregulation of STRE controlled genes or upregulation of ribosomal gene expression (see Chapter I: 'Regulation of ribosomal protein synthesis'), are maintained during growth, suggesting the existence of additional mechanisms that may control PKA activity. An important detail, in this context, is the fact that PKA activation following addition of glucose to derepressed cells does only occur in the presence of a complete, rich growth medium. When essential nutrients (*e.g.*, nitrogen, phosphor, sulfur) are missing, cells arrest in stationary phase G_0 and acquire characteristics of low PKA activity, even if there is abundant glucose available. Readdition of the essential nutrient to such starved cells, in the presence of glucose, induces activation of PKA (Thevelein, 1984a; Thevelein and Beullens, 1985; Hirimburegama *et al.*, 1992) without an increase in intracellular cAMP (Durnez *et al.*, 1994). The conclusion of these experiments was that there exists an additional pathway, besides the Ras/cAMP pathway, that controls PKA activity and that requires both glucose and all essential nutrients for activation. Based on these findings, this putative pathway was named 'fermentable growth medium' pathway (FGM) (Thevelein, 1994). The existence of this pathway is supported by the fact that activation of PKA by addition of nitrogen to nitrogen-starved cells has some particular features, *i.e.* it is independent of phosphorylation of the fermentable sugar (Pernambuco *et al.*, 1996) and independent of the presence of the PKA regulatory subunit Bcy1 (Thevelein, 1994).

Furthermore, while inactivation of the G protein coupled receptor system Gpr1/Gpa2 abolishes glucose-induced cAMP synthesis, glucose-induced PKA activation including the associated phenotypes are delayed, but not prevented (Colombo *et al.*, 1998; Kraakman *et al.*, 1999). This supports the hypothesis that activation of PKA or one of its targets, notably Rim15 (see below), is not only due to glucose-induced cAMP synthesis.

The only component of the FGM pathway identified so far is the protein kinase Sch9 (Crauwels *et al.*, 1997). It was originally isolated in a screen for suppressors of the conditional growth defect of a temperature sensitive *cdc25* strain (Toda *et al.*, 1988). Sch9 is homologous to Tpk proteins. Overproduction of Sch9 rescues the lethality caused by loss of the three Tpks and produces similar phenotypes as overproduction of Tpks, including increased sensitivity towards various stresses (Crauwels *et al.*, 1997). Loss of Sch9, on the other hand, causes a slow growth phenotype that can be suppressed by increased PKA activity (*e.g.* as a consequence of deletion of *BCY1* or expression of $RAS2^{Val19}$). Sch9 may function in a pathway that is partially redundant with the cAMP/PKA pathway, since it has been shown that Sch9 is not required for glucose-induced activation of the Ras/cAMP pathway. Interestingly, activation of PKA targets following readdition of nitrogen to nitrogen-starved cells was found to be independent of the presence of Gpr1 and Gpa2, while being strongly dependent on the presence of Sch9. Thus, Sch9 may act as part of a putative FGM signaling pathway that feeds into PKA via a yet poorly understood cAMP- independent mechanism.

Physiological processes under the control of PKA

In order to respond adequately to a change in the environment, the yeast cell has to alter its metabolic activities. In the case of the cAMP/PKA pathway, the cells sense one or several different stimuli and transmit the signal to PKA, which then controls cellular events such as carbohydrate metabolism, stress protection, growth and cell cycle progression. The means by which this is achieved is still matter of investigation and elucidation of the involved mechanisms will certainly provide a better understanding of how cells coordinate growth and metabolic activities in response to extracellular events. Current knowledge on the different targets of PKA and their involvement in cellular physiology is outlined in the following sections (see also Fig. 3).

Gluconeogenesis and glycolysis

One role of the cAMP/PKA pathway is to signal the presence of glucose in cells growing on non-fermentable carbon sources. The adaptations in metabolism that allow the switch from gluconeogenesis to fermentation include glucose repression via the Snf1/Mig1 pathway (Ronne, 1995). In addition, cAMP-dependent processes have been identified as being crucial for this switch (Holzer, 1984; Thevelein, 1984b). The gluconeogenic enzyme fructose-1,6-bisphosphatase Fbp1, for instance, is inactivated in a PKA-dependent, reversible manner (Muller and Holzer, 1981; Mazon *et al.*, 1982; Rose *et al.*, 1988). In parallel, phosphofructokinase 1 is rapidly activated by PKA-dependent phosphorylation. Both adaptations help to increase the flow of metabolites through the glycolytic pathway (Francois *et al.*, 1984). Moreover, cAMP-dependent transcriptional regulation of enzymes involved in carbon metabolism has been described. The transcriptional activator Adr1, which is inactivated transcriptionally and posttranscriptionally by PKA, controls the expression of alcohol dehydrogenase II (needed for the use of ethanol as carbon source by the yeast), (Denis and Gallo, 1986; Cherry *et al.*, 1989). As a result, activation of PKA assists the main glucose repression pathway to switch from gluconeogenesis to glycolysis.

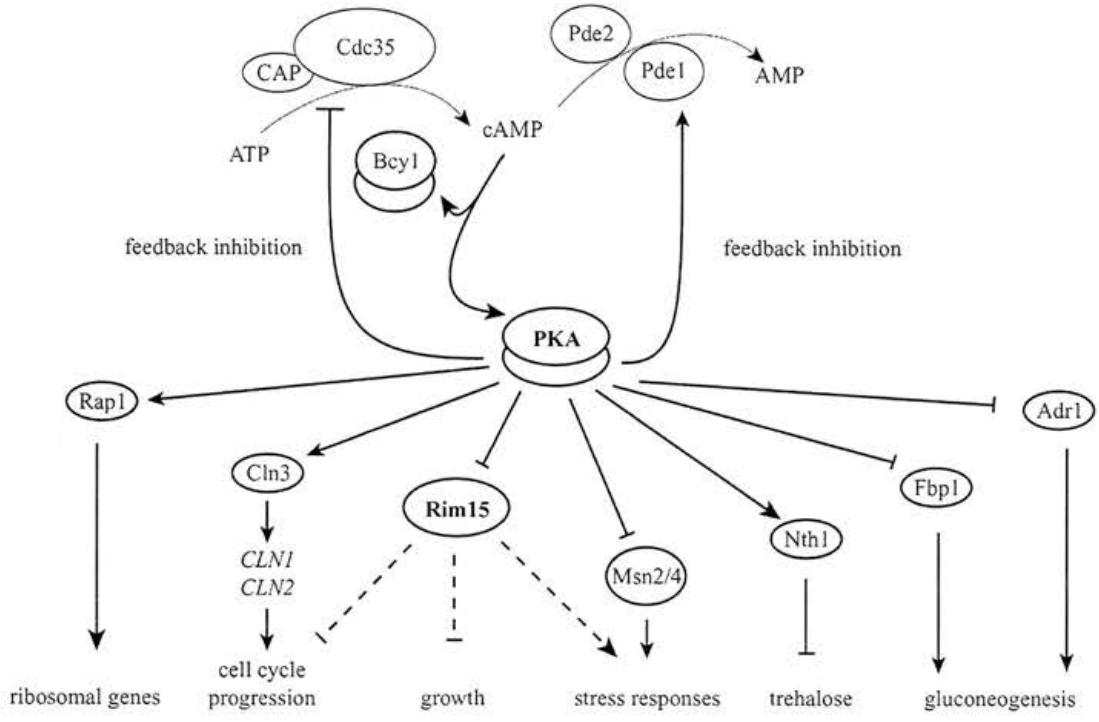

Fig. 3 PKA and its most important targets. For details see legend of Fig. 1.

Trehalose

Trehalose (α-D-glucopyranosyl-1,1-α-glucopyranoside) has been found in a wide range of different organisms ranging from bacteria to fungi, plants and lower animals (Elbein, 1974; Thevelein, 1984b; Crowe *et al.*, 1992). Many of these organisms are anhydrobiotic, which means that they are capable of withstanding extreme drought stress and to recover upon rehydration. In the dry state, these organisms contain high amounts of trehalose and resumption of metabolic activity is accompanied by degradation of trehalose. It could be shown that the role of this disaccharide in helping the cells to survive adverse conditions like water loss, is in acting as a stress protectant (Crowe *et al.*, 1984; Warner, 1989; Colaco *et al.*, 1994). Two functions for trehalose have been proposed, namely stabilization of biological membranes as shown by its capability to prevent leakage or fusion of lipid compartments normally observed upon water depletion (Crowe *et al.*, 1985), and protection of proteins (Colaco *et al.*, 1994). The mechanism by which this is achieved is still a matter of debate, but it has been demonstrated in several instances that this sugar protects the native conformation of proteins during conditions of heat, freezing and desiccation (Carpenter *et al.*, 1987; Singer and Lindquist, 1998), that it preserves the activity of enzymes (Hottiger *et al.*, 1987a; Hottiger *et al.*, 1987b; Hottiger *et al.*, 1994; Sola-Penna and Meyer-Fernades, 1994), and that it reduces aggregation of already denatured proteins, a task originally assigned exclusively to heat shock proteins (Attfield, 1987; Plesofsky-Vig, 1996).

Also yeast is known to accumulate trehalose under stress conditions or in stationary phase following depletion of glucose (Lillie and Pringle, 1980). It is synthesized in a two-step reaction. In a first step, trehalose-6-phosphate (T6P) is formed from glucose-6-phosphate and UDP-glucose, catalyzed by the enzyme T6P synthase, which is encoded by *TPS1*. In a subsequent reaction, the phosphate is cleaved off by a highly specific phosphatase encoded by *TPS2* (Bell *et al.*, 1992; Vuorio *et al.*, 1993). The two proteins are associated in a complex together with the two regulatory subunits Tps3 and Tsl1 (Reinders *et al.*, 1997; Bell *et al.*, 1998). Degradation of trehalose is catalyzed by trehalase, of which two forms have been found. The acid trehalase, encoded by *ATH1,* is found in the vacuole, whereas the neutral trehalase, encoded by *NTH1*, is found in the cytosol, the location of trehalose storage (Kopp *et al.*, 1993; Van Dijck *et al.*, 1995; Nwaka and Holzer, 1998). The role of acid trehalase is not clearly understood, but it seems to be important for growth on trehalose as a carbon source (Nwaka *et al.*, 1996).

Under various different stress conditions, it has been shown that synthesis and catabolism of trehalose are correlated with low and high PKA activity, respectively. Accordingly, low levels of PKA in diauxic or stationary phase cells was found to be associated with high stress resistance and high trehalose levels, which result from inactivation of the neutral trehalase and stimulation of the T6P-synthase (Uno *et al.*, 1983; Thevelein and Beullens, 1985; Panek *et al.*, 1987). In contrast, high levels of PKA during phases of rapid growth, or after addition of a fermentable sugar to starved cells were found to stimulate the degradation of trehalose as a result of rapid activation of neutral trehalase with concomitant inhibition of the T6P-synthase. Interestingly, degradation of trehalose after recovery from stress is as important as accumulation during the stress phase, since *nth1* mutants that have a trehalose degradation defect, were found to be impaired in recovering from stress. It seems that high concentration of trehalose may inhibit the reactivation of denatured proteins by HSPs (Singer and Lindquist, 1998).

Various results suggest that activation of trehalase occurs by direct phosphorylation of neutral trehalase (Uno *et al.*, 1983; Thevelein, 1984b; Toda *et al.*, 1985; App and Holzer, 1989; Nwaka and Holzer, 1998), but the precise phosphorylation sites have not yet been identified (Kopp *et al.*, 1993; Kopp *et al.*, 1994; Wera *et al.*, 1999). Notably, in addition to posttranslational control of neutral trehalase, the PKA pathway has also been implicated in transcriptional control of trehalose metabolism genes via STRE elements (Winderickx *et al.*, 1996, see below).

Stress response

Microorganisms like *S. cerevisiae* live in a highly variable environment. Conditions like temperature, osmolarity or nutrient availability are far from constant. It is therefore of special importance for these organisms to rapidly adapt to these various stress conditions. As a consequence, they have developed molecular responses that allow repair of damages (*e.g.* via production of heat shock proteins [HSPs]), and protect against further damage (*e.g.* via synthesis of trehalose) during severe stress. Yeast cells exposed to a mild stress, in contrast, can adapt to support higher doses of a given stress, and eventually develop induced tolerance towards other forms of stress. This phenomenon, referred to as cross-protection, suggests that there is a common mechanism that integrates the different forms of stress into a general stress response. This idea was supported by the finding, that the expression of several heat shock

proteins could be induced by different stresses (Kurtz *et al.*, 1986; Werner-Washburne *et al.*, 1989).

The discovery of a common *cis*-regulatory element in the promoters of stress-induced genes was a major breakthrough in this area of research. This element was initially identified in the promoters of *CTT1* (Wieser *et al.*, 1991), encoding cytoplasmic catalase T, and of the DNA-damage response gene *DDR2* (Kobayashi and McEntee, 1990). This element is able to mediate transcriptional induction following different forms of stress, and was therefore named STRE (stress responsive element). STRE sequences with the core consensus sequence AGGG (Ruis and Schuller, 1995) are found in the promoters of many genes, including the trehalose biosynthesis genes *TPS1*, *TPS2*, *TPS3* and *TSL1* (Gounalaki and Thireos, 1994; Winderickx *et al.*, 1996), the glycogen synthase and a glycogen phosphorylase genes *GSY2* and *GPH1*, respectively, the heat shock genes *HSP12*, *HSP104* and *HSP26*, the polyubiquitin encoding gene *UBI4* (Tanaka *et al.*, 1988; Moskvina *et al.*, 1998) and the neutral trehalase gene *NTH1* (Zahringer *et al.*, 2000). Analysis of the complete yeast genome revealed the presence of 186 potential STRE-regulated genes (Treger *et al.*, 1998). A single copy of this element is sufficient to activate a reporter gene by various stress conditions, but several copies in either direction, as it is found in most genes, greatly enhance the stimulatory effect (Kobayashi and McEntee, 1993; Alepuz *et al.*, 1997). Nevertheless, presence of a STRE-like sequence does not necessarily imply functionality of the element and not all STRE-containing promoters confer the same pattern of expression to corresponding genes. The different expression patterns between STRE-containing genes may be explained by the promoter context in which the STREs are found. One special case is the promoter of *SSA3*, which codes for a member of the Hsp70 protein family. It contains a *cis*-element which resembles STRE and which is responsible for the induction of *SSA3* during the post-diauxic phase upon nutrient limitation. Nevertheless, this element does not confer inducibility by other stresses like heat shock (Boorstein and Craig, 1990), or osmotic stress, both conditions that are known to induce STRE-driven transcription (Winderickx *et al.*, 1996). Interestingly, the consensus site of this element was identified as (T/A)AGGGA, which is similar to the AG_4 STRE, and which was named post-diauxic shift (PDS) element (Boorstein and Craig, 1990). Thus, expression of stress-induced genes is modulated by a combination of regulatory promoter elements, which can exhibit redundant or additive activatory effects depending on the promoter context and the applied stress condition.

Two transcription factors, Msn2 and Msn4, have been identified to be involved in STRE-mediated gene expression (Estruch and Carlson, 1993; Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). Both of them have been shown to recognize and bind STRE *in vitro* and *in vivo* (Martinez-Pastor *et al.*, 1996; Gorner *et al.*, 1998), and to be required for induction of a STRE-*lacZ* reporter fusion in response to nutritional starvation, heat shock, oxidative stress and osmotic shock (Martinez-Pastor *et al.*, 1996). A *msn2Δmsn4Δ* double mutant shows reduced expression of STRE-controlled genes like *CTT1*, *HSP12* and *DDR2* under stress conditions. Nevertheless, a significant induction of these genes upon osmotic and oxidative stress is independent of Msn2 and Msn4, indicating either the involvement of additional STRE-binding factors that can be specifically activated under those stress conditions, or the presence of additional *cis* regulatory sequences in the promoters of these genes.

Genetic evidence suggests that the two proteins Msn2 and Msn4 are functionally redundant (Estruch and Carlson, 1993), although more recent experiments show that they could have different roles in regulation of stress response (Treger *et al.*, 1998). In an analysis of Msn2- or Msn4-dependent transcription following heat-shock, some genes such as *PGM2* were found to have lower expression levels in the absence of Msn2 alone, while the expression of others, like *PDE2,* was not affected by the absence of Msn2 alone, but significantly reduced in the absence of both Msn2 and Msn4. The finding that Msn2 and Msn4 have different accumulation patterns at the diauxic transition (DeRisi *et al.*, 1997) supports the notion that they do only partially overlap in function and suggests that they have, yet to be discovered, unique functions in transcriptional regulation.

Interestingly, it could be shown that there is a correlation between STRE-induced expression of stress genes and the activity of the Ras/cAMP pathway (Kobayashi and McEntee, 1993; Marchler *et al.*, 1993). Accordingly, STRE-dependent transcription is enhanced in cells with low PKA activity, whereas it is reduced in cells with constitutive high levels of PKA activity. Thus, stress-induced gene expression seems to be negatively regulated by the Ras/PKA pathway, which thereby counteracts expression of stress genes under conditions of optimal growth. Experiments using epitope-tagged proteins and green fluorescent protein (GFP)-fusions revealed that the subcellular localization of Msn2/Msn4 could be a possible way how transcription is controlled by the PKA pathway (Gorner *et al.*, 1998). In unstressed cells, Msn2 and Msn4 are distributed throughout the cytoplasm and partly excluded from the nucleus. Upon exposure to stress conditions, Msn2 and Msn4 rapidly translocate to the nucleus and it was found that this translocation is correlated with low PKA activity.

Accordingly, in cells with attenuated PKA activity, these factors are constitutively located in the nucleus. Recent results suggest that direct phosphorylation of the Msn2 nuclear localization signal (NLS) domain by PKA may be crucial for control of subcellular localization (Gorner *et al.*, 2002). A Msn2-NLS domain fused to GFP showed Msn2-like shuttling upon stress induction. Serine to aspartate mutations in the four PKA phosphorylation consensus sites (RRXS) found in the NLS, mimicking serine phosphorylation, prevented the protein from accumulation in the nucleus, whereas serine to alanine mutations led to constitutive nuclear localization. Thus, PKA-mediated phosphorylation of the NLS domain may prevent nuclear localization of Msn2 during phases of high PKA activity.

Recently, it was shown that nuclear localization of Msn2/Msn4 is not solely controlled through PKA. It has been shown that rapamycin, that acts through the TOR pathway, induces nuclear localization of Msn2 and Msn4 (Beck and Hall, 1999). This effect may be due to a release of Msn2 and Msn4 from the cytoplasmic anchor protein Bmh2, which retains these factors in the cytoplasm. Since release of Msn2/Msn4 from Bmh2 can also be induced by glucose depletion, it may be possible that the release from Bmh2 is a process that is regulated by both, the TOR and the Ras/cAMP pathway. Another mechanism involved in STRE-regulation is the nuclear export of Msn2 and Msn4 transcription factors via the export receptor Msn5 (Alepuz *et al.*, 1999). While Msn2 and Msn4 are constitutively located in the nucleus in a *msn5Δ* mutant, regulation of STRE-dependent genes is not affected (Estruch, 2000). Thus, additional mechanisms must be involved in transduction of the stress signal, other than control of subcellular localization.

Regulation of ribosomal protein synthesis

Rapidly growing exponential phase cells require elevated levels of protein synthesis. Accordingly, it has been shown that these cells contain a two- to five-fold higher amount of ribosomes than stationary phase cells (Warner, 1989). Synthesis of ribosomes is mainly controlled on the transcriptional level of the more than 70 ribosomal genes dispersed throughout the genome. Almost all of them contain one or more recognition sites for the transcription factor Rap1 in their promoter region (Teem *et al.*, 1984). Rap1 is an abundant nuclear protein, and its recognition site is found also in a variety of genes, that are unrelated to ribosomal genes. Depending on the promoter context, Rap1 acts as a transcriptional activator or repressor. For the ribosomal protein genes, Rap1 acts exclusively as an activator.

Transcriptional activation of some of the ribosomal genes like *RPL16*, *RPL25* and *RPL29* have been shown to be dependent on cAMP (Klein and Struhl, 1994; Neuman-Silberberg *et al.*, 1995). The induction in transcription of these genes is dependent on the Rap1 binding sites. Consequently, it has been shown that constitutively active PKA increases Rap1-dependent transcription of ribosomal genes (Klein and Struhl, 1994). Moreover, Rap1 protein is degraded in cells with low levels of cAMP, suggesting that PKA could control ribosomal gene expression through modulating Rap1 levels rather than, or in addition to, direct phosphorylation (Neuman-Silberberg *et al.*, 1995), but the precise mechanism is still unknown.

Feedback inhibition of the Ras/cAMP pathway by PKA

The Ras/cAMP pathway is subjected to feedback inhibition through PKA (Nikawa *et al.*, 1987a). Strains with attenuated PKA activity show hyperaccumulation of cAMP, whereas strains with constitutive PKA activity have low cAMP levels. Many targets of this feedback control have been proposed, and involve Cdc25 (Munder and Kuntzel, 1989), Ras2 (Resnick and Racker, 1988), Ira1/2 (Tanaka *et al.*, 1989; Tanaka *et al.*, 1990b) and adenylate cyclase (De Vendittis *et al.*, 1986), but the most promising candidate for a physiologically relevant target seems to be a phosphodiesterase.

As described, the yeast genome encodes two phosphodiesterases Pde1 and Pde2 with different affinities for their common substrate cAMP. The high-affinity phosphodiesterase Pde2 controls the cAMP level around its basal, physiological level (*i.e.* 1 mM). The low-affinity phosphodiesterase Pde1, in contrast, has a K_m around 50 mM which is considerably higher than the estimated intracellular cAMP level (1 mM). Accordingly, Pde1 seems to be involved in the breakdown of the high, but transient cAMP levels observed upon glucose-stimulation. In line with this notion, deletion of *PDE1* causes a huge increase in the glucose-induced cAMP signal, whereas overexpression of Pde1 eliminates this cAMP signal. In a strain with reduced PKA activity on the other hand, the phosphodiesterases are unable to prevent hyperaccumulation of cAMP, indicating that PKA activity is required for efficient breakdown of cAMP by the phosphodiesterases (Thevelein, 1992). In addition, a putative PKA phosphorylation site has been identified in Pde1 and mutation of the conserved serine at position 252 to an alanine results in the same huge increase in the glucose-induced cAMP signal as loss of Pde1 (Kennelly and Krebs, 1991; Ma *et al.*, 1999). Notably, The serine 252

to alanine mutation has no effect on the enzyme activity *in vitro*, indicating that PKA-dependent phosphorylation of Pde1 is specifically required for its function *in vivo*.

Cell cycle and growth

A yeast cell has to coordinate its cell cycle with growth in response to growth conditions. The different stages of the yeast cell cycle include G_1, S (for DNA synthesis) G_2 (an intermediary growth phase associated with chromatin condensation), and M (for mitosis and cytokinesis). After mitosis, the daughter cell enters G_1, where it increases in size until the start of the next cell cycle. Once a cell starts a new cycle, it has to complete it until it reaches again G_1; it can not exit at another point to enter a quiescent state. Therefore, the cell cycle contains a nutrient checkpoint at which the cell has to meet several criteria in order to pass through and continue the cycle. The nutrient control checkpoint is at the end of G_1 and is called START (Jagadish and Carter, 1977; Johnston *et al.*, 1977). At this point, the cell has to decide, based on the availability of nutrients and the environmental situation, whether to start another cycle or to enter a quiescent state G_0, also referred to as stationary phase. On a molecular level, these decisions are coordinated by cyclins and the cyclin-dependent protein kinase Cdc28 (CDK). Different cyclins, which are synthesized and degraded in a cell cycle-dependent manner associate with Cdc28, modulating its kinase activity and substrate specificity and thereby triggering stage-specific events. Amongst these, the Cln1-Cdc28 and Cln2-Cdc28 kinase complexes are involved in a process that allows the cell to pass through the nutrient control point of START (Tyers *et al.*, 1993; Epstein and Cross, 1994; Di Como *et al.*, 1995; Stuart and Wittenberg, 1995).

It has been found early on that the Ras/cAMP pathway plays a crucial role in regulating cell cycle progression, particularly since two components of the pathway, Cdc25 and Cdc35, have originally been identified as cell division cycle mutants that arrest in $G_{1/0}$ at the restrictive temperature (Hartwell, 1974; Hartwell *et al.*, 1974). This arrest is observed generally upon inactivation of the Ras/cAMP pathway following nutrient starvation (Matsumoto *et al.*, 1985) and is associated with loss of cyclin synthesis (Hubler *et al.*, 1993). Accordingly, it could be shown that cAMP is necessary for expression of *CLN1* and *CLN2*. Additionally, this response is inhibited by deletion of *CLN3* and Cln3-Cdc28 activity is regulated in correspondence to carbon source availability (Hall *et al.*, 1998). Although *CLN3* expression itself is not regulated by PKA, Cln3-Cdc28 activity is enhanced by active PKA during growth on a

fermentable carbon source. Also, overexpression of *CLN3* is sufficient to bypass the requirement of cAMP for progression through START, indicating that Cln3 may be a target of PKA. It is not clear, however, whether PKA is required for Cln3-Cdc28 activity or whether PKA is only required for synthesis of the cyclins (Thevelein and de Winde, 1999). Moreover, it has not been shown to date that progression over START is dependent on an increase in cAMP levels or in PKA activity (Thevelein, 1991). Notably, starvation of yeast cells for nitrogen causes arrest at START although nitrogen starvation does not affect cAMP levels. On the other hand, cells with a hyperactive Ras/cAMP pathway do not arrest at G_1 upon nutrient depletion, but arrest rather randomly in the cell cycle, probably when intracellular storage carbohydrates are used up (Tatchell, 1993; Markwardt *et al.*, 1995). The function of the Ras/cAMP pathway is probably to provide a basal cAMP level, which is one of the requirements for START, rather than an acting as a second messenger for progression over START.

Protein kinase Rim15

The protein kinase Rim15 was first identified as a stimulator of meiotic gene expression (Vidan and Mitchell, 1997). It was then isolated in a two-hybrid screen using Tps1 as bait that aimed at identifying regulatory proteins of T6P synthesis (Reinders *et al.*, 1998). Analysis of the *rim15* mutant revealed, that Rim15 was not only required for induction of trehalose synthesis in cells grown to stationary phase, but also for various other cellular responses to nutrient starvation. A strain deleted for *RIM15* had defects in glycogen accumulation, in transcriptional derepression of stress regulated genes such as *HSP12*, *HSP26*, and *SSA3*, in induction of thermotolerance, in starvation resistance, and in proper G_1 arrest upon nutritional depletion. All these effects resemble the phenotypes of a hyperactive Ras/cAMP pathway. It was therefore suggested that Rim15 may act in the Ras/cAMP pathway, being inactive during exponential growth and required for entry into stationary phase. The presence of five consensus sites for PKA phosphorylation, RRXS (Edelman *et al.*, 1987) further suggested Rim15 may be a direct target of PKA. This hypothesis was supported by several experiments. Deletion of *RIM15* could suppress the lethal effect of complete loss of PKA. Moreover, loss of Rim15 was also epistatic over *tpk* mutations with respect to various stationary-phase-associated phenotypes. Conversely, overexpression of Rim15 exacerbated the growth defect of strains with attenuated PKA activity and overexpression mimicked a nutrient limited state

in exponentially growing wild-type cells, which includes elevated trehalose levels, derepression of *SSA3* and enhanced thermotolerance. Finally, it could be shown that PKA indeed phosphorylates Rim15 *in vitro* and thereby reduce Rim15 protein kinase activity (Reinders *et al.*, 1998). As expected, mutation of the five PKA consensus sites in Rim15 rendered the kinase insensitive to negative regulation by PKA. Taken together, these results place Rim15, as the first protein kinase directly downstream of PKA. According to the present model it is under negative control of the Ras/cAMP pathway and positively controls a subset of G_0 related events following release from PKA inhibition under glucose starvation conditions.

Chapter II

Identification of downstream targets of protein kinase Rim15

Identification of downstream targets of the protein kinase Rim15

Introduction

It is of great interest to understand how cells control growth and cell cycle progression in response to extracellular triggers, since already small alterations in this tight control system can lead to unregulated growth and cancer formation. In *Saccharomyces cerevisiae,* it has been shown that the Ras/cAMP pathway controls growth and proliferation with respect to nutritional conditions. Inactivation of the pathway upon nutrient depletion leads to cell cycle arrest at G_1 and entry into a state named stationary phase, or G_0, which is characterized by enhanced stress and starvation tolerance. Despite the remarkable progress made in how the components of the Ras/cAMP pathway activate cAMP dependent protein kinase (PKA) in response to nutrient availability, the molecular events that lead to PKA-dependent cell cycle arrest or growth control remain poorly understood. The protein kinase Rim15, recently identified in our lab, controls a subset of PKA-dependent adaptations to nutrient starvation. Rim15 is involved in activation of trehalose and glycogen synthesis, derepression of several stress-induced genes, and negative control of cell cycle progression when it is released from inhibition of PKA upon entry into post-diauxic shift phase. Under starvation conditions, *rim15Δ* cells continue to divide and eventually die, probably as a consequence of lack of carbohydrate storage. Thus, Rim15 is an important protein that negatively controls growth and cell cycle progression, and identification of downstream targets of Rim15 may yield new insights to the mechanisms of cell proliferation control.

Results

Nutrient-limitation induced *STRE* expression is largely independent of Rim15

It has been shown that the protein kinase Rim15 controls a subset of PKA regulated phenotypes (Reinders *et al.*, 1998). One of these is the transcriptional derepression, upon nutrient starvation, of various genes that are known to be controlled by the transcription factors Msn2/4 (Boy-Marcotte *et al.*, 1998). One possible model may therefore be that Rim15 directly activates these transcription factors. To test this hypothesis, we looked at expression of a reporter *lacZ* construct under the control of the *HSP26* promoter. *HSP26* is one of the

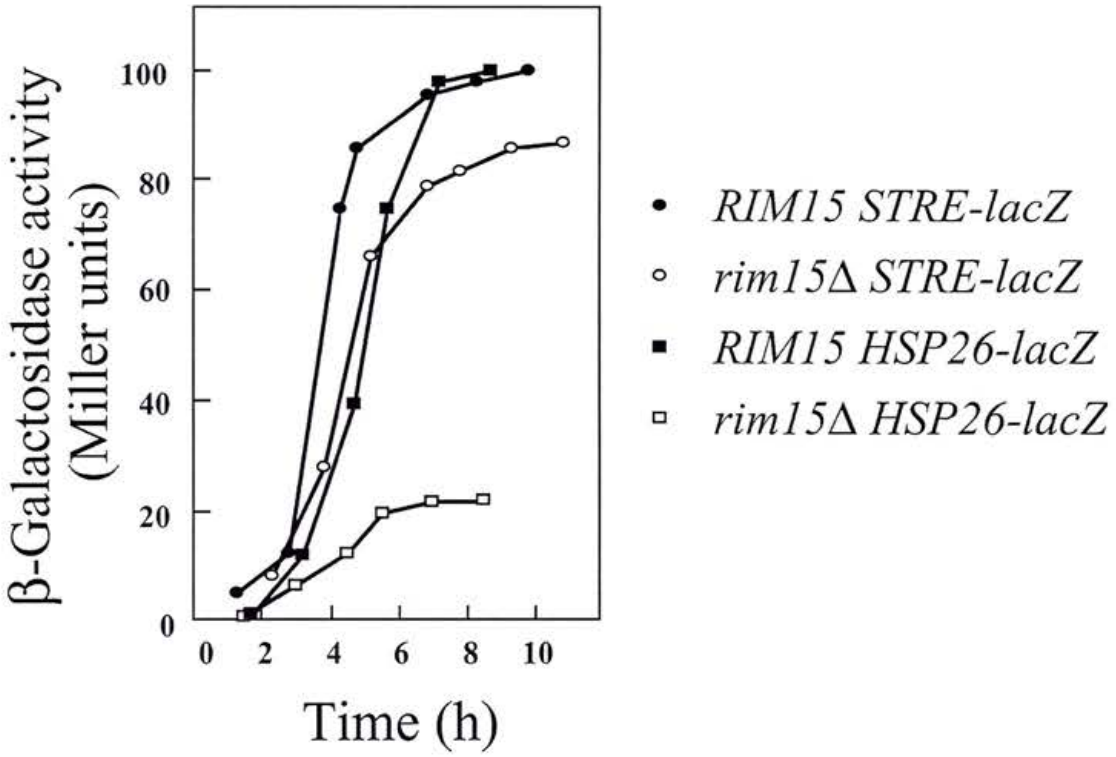

Fig. 1 Wild-type (YEF473: *RIM15*) and mutant (AR2: *rim15Δ*) cells carrying a STRE-*lacZ* or a *HSP26-lacZ* reporter construct were grown on SD medium containing 1% glucose and followed during entry into the diauxic shift phase. β-galactosidase activities were measured (as descibed in Miller, 1972) at the times indicated to monitor the induction of the reporter genes upon nutrient limitation.

genes, the expression of which has been shown to be dependent on Msn2/4 during the diauxic transition (Boy-Marcotte *et al.*, 1998). We found that expression of the *HSP26* reporter was reduced in a *rim15Δ* strain during the diauxic shift (Fig. 1), which is similar to the defect in *msn2Δmsn4Δ* double mutants. However, activation of the *STRE-lacZ* reporter during the same growth phase – *STRE* is the element recognized by the Msn2/4 transcription factors (Martinez-Pastor *et al.*, 1996) – was only slightly reduced in a *rim15Δ* mutant, when compared to a *msn2Δmsn4Δ* double mutant (data not shown). Thus, nutrient limitation-induced *STRE*-expression is largely independent of Rim15, indicating that Rim15 may not act through the transcription factors Msn2 and Msn4. In addition, the result obtained with *HSP26-lacZ* indicates that there must be additional regulatory elements in stress-induced genes that are regulated in a Rim15-dependent manner.

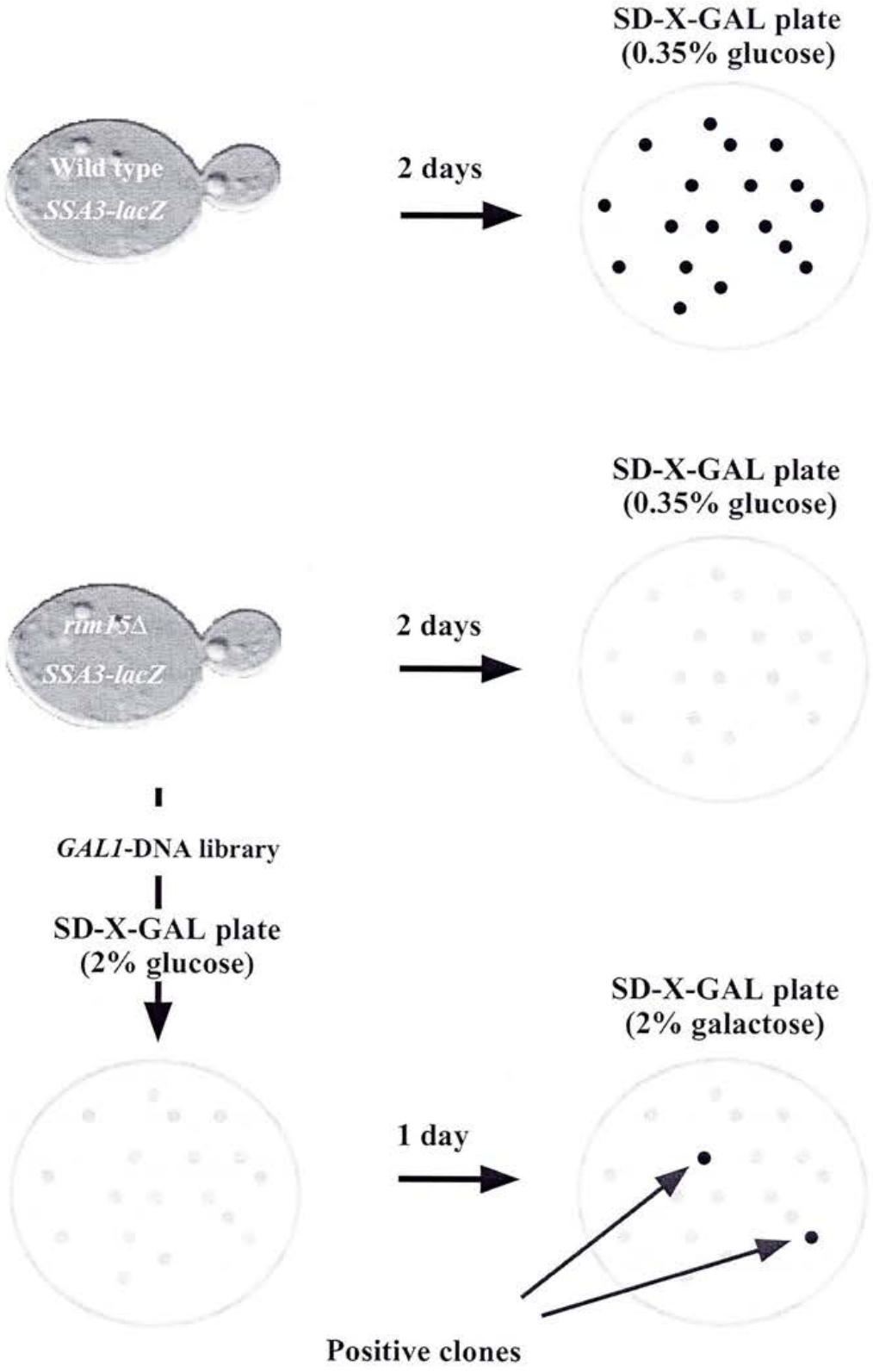

Fig. 2 Schematic of the screen setup for identification of dosage suppressors of the defect of a *rim15Δ* mutant in derepression of *SSA3*.

Screen for dosage suppressors of *rim15Δ*

In order to identify downstream targets of Rim15, we initiated a screen for dosage suppressors of the phenotypic defect of a *rim15Δ* mutant in inducing expression of *SSA3* upon nutrient limitation. We chose the heat shock protein promoter *SSA3* for our reporter system, since nutrient-limitation-induced derepression of *SSA3* has been shown to be independent of Msn2 and Msn4 (Martinez-Pastor *et al.*, 1996), but to be strongly dependent on Rim15 (Reinders *et al.*, 1998).

Cells of a wild-type strain transformed with plasmid pWB204Δ-236, containing the reporter gene *lacZ* under the control of the *SSA3* promoter (Boorstein and Craig, 1990), form blue colonies after 2 days on 0.35% glucose plates supplemented with the β-galactosidase substrate X-gal. The optimal glucose concentration to ensure that glucose is the limiting nutrient when cells become starved on the plates was determined in control experiments. Cells of a *rim15Δ* strain, as expected, form white colonies, since they are defective in induction of *SSA3* promoter-driven transcription (Fig. 2). To screen for dosage suppressors of this particular defect of *rim15Δ* mutants, we used a yeast genomic library under the control of the strong inducible *GAL1* promoter (Ramer *et al.*, 1992). We tested the system by introducing *RIM15* under the control of the *GAL1* promoter into the *rim15Δ* strain carrying the *SSA3-lacZ* reporter plasmid. As expected, shifting cells from glucose to galactose plates resulted in the formation of blue colonies, showing that overexpression of *RIM15* complements the loss of *RIM15*.

Transformants were first grown under non-inducing conditions on selective glucose plates for 2 days, thereby preventing a selective advantage or disadvantage of growth-related genes. The colonies were then replica plated on selective plates containing galactose and X-gal. After one day on galactose, 168 putative positive clones were picked from around 2.5×10^6 colonies screened. The colonies were restreaked on glucose to propagate the strains under non-inducing conditions. The patches were then replica plated on galactose/X-gal plates to verify *SSA3-lacZ* induction upon overproduction of the library encoded proteins. Subsequently 75 clones remained positive and the genomic library insert-bearing plasmid were rescued by isolation and passage through an *E. coli pyrF*⁻ strain, that allows selection for *URA3*⁺ plasmids. To confirm that the gene products on the isolated plasmids were indeed the *rim15Δ*-suppressing agents that induced *SSA3* transcription on galactose and to see if this effect was

reproducible, the plasmids were retransformed into the *rim15Δ* strain contain the *SSA3-lacZ* reporter. This procedure narrowed down the number of positive clones to 48. All these plasmids were sequenced with a primer annealing in the *GAL1* promoter that allows sequencing across the junction between the *GAL1* promoter and the fused library insert. The sequences were compared to the Saccharomyces Genome Database (SGD, Cherry *et al.*, 1998) using BLAST and information on the identified open reading frames was retrieved from the Yeast Protein Database (YPD, Hodges *et al.*, 1999). Table I summarizes these findings.

Table I. Isolated clones from the *rim15Δ* dosage suppressor screen

Name	Description	Cellular Role
ACT1 (2 times)	actin	involved in cell polarization, endocytosis, and other cytoskeletal functions
ASE1	microtubule-associated protein	required for anaphase spindle elongation
ATP11	F1-ATP synthase assembly protein	complex assembly protein
BNI1	unknown	involved in cytoskeletal control, required for actin (Act1p) filament assembly and proper bipolar budding pattern
BRN1	subunit of condensin protein complex	required for proper chromosome condensation and segregation
CDH1	protein of the WD (WD-40) repeat family	binds to substrates (*CLB2*, *CLB3*, *CDC5*, *HSL1*) of the anaphase promoting complex (APC) and targets them for degradation
CHRMT (3 times)	mitochondrial DNA	
EBP2	unknown	involved in pre-rRNA processing and ribosomal subunit assembly
GIS1	transcription factor	damage-responsive repressor of *PHR1*, involved in DNA repair
ICY1	unknown	interacts with the cytoskeleton, involved in vesicular transport
MAL1	unknown	defective maltose fermentation
MAL31	maltose permease	carbohydrate metabolism
MAP2	methionine aminopeptidase isoform 2	cleaves N-terminal methionine
MYO4	myosin heavy chain,	required for proper localization of *ASH1* mRNA,

	class V	required for HO expression

Table I. continued

Name	Description	Cellular Role
NUF1	spindle pole body component	determines the spacing between the ends of microtubules and the central plaque
PDX3	pyridoxine phosphate oxidase	involved in regulating sterol uptake
PHO8	vacuolar alkaline phosphatase	carries out dephosphorylation of phosphopeptides
PKC1	protein kinase C	regulates MAP kinase cascade involved in regulating cell wall metabolism
PMC1	vacuolar Ca2+-transporting P-type ATPase	functions to pump Ca2+ into the vacuole
POP1	protein component of both RNase P and RNase MRP	involved in both tRNA maturation (RNase P) and in 5.8S rRNA processing (RNase MRP)
RDN	ribosomal DNA	
RRN6	component of RNA polymerase I core transcription factor	essential for initiation of RNA polymerase I
SMP1	transcription factor of the MADS box family	pol II transcription
TYE7	basic helix-loop-helix transcription factor	suppresses the Gcr1p requirement for glycolytic gene expression
YDL148C	nuclear and nucleolar protein	involved in 40S ribosomal subunit biogenesis and 18S rRNA maturation
YDR100W (2 times)	unknown	protein of unknown function
YER188W	unknown	protein of unknown function
YFL002W-A (3 times)	Ty2 Transposon	
YGL046W	unknown	protein of unknown function
YGR089W (2 times)	unknown	protein of unknown function
YJL084C	unknown	protein of unknown function
YKR088C	unknown	protein of unknown function
YLR030W	unknown	protein of unknown function
YLR046C	unknown	putative glycosyl-phosphatidylinositol (GPI)-protein
YNL094W	unknown	protein of unknown function

YNL335W	lyase	induced after DNA alkylation damage

Table I. continued

Name	Description	Cellular Role
YOL045W (2 times)	serine/threonine protein kinase	protein of unknown function
YPL041C	unknown	protein of unknown function
YPL098C	unknown	protein of unknown function
ZDS1	putative transcription factor	regulates *SWE1* and *CLN2* transcription, involved in Sir3p phosphorylation, rDNA recombination and silencing, and life span control

Summary of the isolated sequences of the *rim15Δ* dosage suppressor screen. Information about the biochemical function of the gene products as well as their putative or known cellular function was retrieved from the Yeast Protein Database (YPD, Hodges *et al.*, 1999).

A look at the isolated gene products showed that we found almost no redundancy in the isolated plasmids. Moreover, the gene products listed above consist of a broad range of proteins of different biochemical classes involved in a variety of known cellular processes, besides some lesser characterized proteins and open reading frames of completely unknown function. This made it difficult to decide for, or focus on, a specific protein for further characterization. Therefore, we decided to assess whether the clones could suppress additional phenotypes of a *rim15Δ* mutant and whether they may be implicated in growth regulation in a PKA-dependent manner. One additional phenotype of loss of *RIM15* is that cells have a severe defect in sporulation (Reinders *et al.*, 1998). We investigated the effect of overproduction of the isolated genes on sporulation in a *rim15Δ/rim15Δ* strain by counting the sporulation efficiency, which is reduced to less than 1% in comparison to about 30% in the wild type. Of the 48 genes, 4 were able to at least partially restore the sporulation efficiency of *rim15Δ/rim15Δ* cells (Table II). Additionally, we introduced all isolated library plasmids in a strain with attenuated PKA activity. Several clones compromised the growth of a $tpk2^{ts}$ strain at the semi-permissive temperature of 31.5°C when overproduced on galactose, indicating that they may antagonize PKA-dependent growth. The results are summarized in Table II.

Table II. Analysis of the clones from the dosage suppressor screen of *rim15Δ*

Name	Color development on plate	Suppression of sporulation defect	Growth inhibition at 31.5°C in a tpk^{ts} strain	
			Glucose	Galactose
ACT1	weak	no	+	+
ASE1	weak	no	−	−
ATP11	weak	no	−	−
BNI1	weak	no	−	−
BRN1	**strong**	no	−	−
CDH1	**strong**	no	−	+
EBP2	weak	no	−	−
GIS1	**strong**	**yes**	−	+
ICY1	weak	no	−	+
MAL1	weak	no	−	+
MAL31	weak	no	−	−
MAP2	weak	no	+	+
MYO4	**strong**	no	−	−
NUF1	weak	no	−	+
PDX3	weak	no	−	−
PHO8	weak	no	−	−
PKC1	**strong**	**yes**	−	+
PMC1	weak	no	−	+
POP1	weak	no	−	+
RRN6	weak	no	−	−
SMP1	**strong**	no	−	−
TYE7	**strong**	no	−	+
YDL148C	weak	no	+	+
YDR100W	weak	no	−	−
YER188W	weak	no	−	−
YGL046W	weak	no	−	−
YGR089W	weak	no	+	+
YJL084C	weak	no	+	+
YKR088C	weak	no	−	+
YLR030W	weak	no	−	−
YLR046C	weak	no	+	+
YNL094W	weak	no	+	+

Table II. continued

Name	Color development on plate	Suppression of sporulation defect	Growth inhibition at 31.5°C in a *tpk*^{ts} strain	
			Glucose	Galactose
YNL335W	weak	**yes**	−	+
YOL045W	**strong**	no	+	+
YPL041C	weak	**yes**	+	+
YPL098C	weak	no	−	−
ZDS1	**strong**	no	−	+

The isolated clones were tested for their ability to suppress phenotypes of the *rim15Δ* mutant. Derepression of *SSA3* upon nutrient limitation was obtained by replica plating a *rim15Δ/rim15Δ* (AR2) strain containing the reporter plasmid pWB204Δ-236 and the library plasmid from glucose to galactose plates containing X-gal. Induction of *SSA3-lacZ* transcription was judged by eye based on the intensity of β-galactosidase-based blue color development after 1 day. Sporulation was measured in the same strains by counting spores in cultures incubated for 3 days in liquid sporulation medium. Suppression of the sporulation defect was judged positive when the sporulation efficiency exceeded 2%. Transformants of *tpk1Δ tpk2-63 tpk3Δ* (SGY446) cells containing the library plasmids were streaked on glucose- and galactose containing SD plates and incubated for 3 days at the semi-permissive temperature 31.5°C, to assess whether the genes inhibit growth (+), or not (−). Genes, which upon overexpression rescued additional *rim15Δ* phenotypes are shown in bold.

Of the 48 isolated proteins, only 6 were able to rescue additional defects of *rim15Δ* mutants. They were able to rescue the defect of *rim15Δ* mutants in proper G_1 arrest upon nutrient limitation as measured by the amount of budded cells in a batch culture after 4 days (Table III). In addition, 4 proteins were also able to suppress the defect of sporulation that can be observed in *rim15Δ* mutants. In a control, overexpression of *RIM15* from the strong *GAL1* promoter complemented these phenotypes as well. This, together with the results presented above, suggest that the proteins may act downstream of, or in parallel to, the Ras/cAMP-Rim15 pathway to control cell proliferation. Surprisingly, none of the 6 proteins fully restored trehalose accumulation in the *rim15Δ* strain in stationary phase (Table III), indicating that regulation of trehalose metabolism by Rim15 may involve yet other effectors.

Table III. Suppression of various *rim15Δ* defects by *GAL1*-driven expression of isolated genes

	Sporulation efficiency	% of budded cells in stationary phase	Trehalose (g/g protein)
Wild type	7.0%	1.1%	0.268 ± 0.011
rim15Δ	0.0%	25.0%	0.111 ± 0.009
rim15Δ +YCpIF2-*RIM15*	22.0%	1.8%	0.267 ± 0.026
rim15Δ +pYES-R-*CDH1*	0.0%	10.0%	0.121 ± 0.009
rim15Δ +pYES-R-*GIS1*	7.0%	0.9%	0.030 ± 0.003
rim15Δ +pYES-R-*PKC1*	3.5%	2.5%	0.056 ± 0.005
rim15Δ +pYES-R-*TYE7*	0.0%	1.1%	0.062 ± 0.002
rim15Δ +pYES-R-*YNL335W*	5.5%	2.4%	0.126 ± 0.026
rim15Δ +pYES-R-*YPL041C*	5.5%	3.0%	0.144 ± 0.034

Sporulation after 3 days in liquid sporulation medium (1% KAc) and the percentage of budded cells in stationary phase (87 hrs on YPD) was determined by microscopic examination of at least 200 cells. Trehalose concentrations in stationary phase cells (87 hrs on YPD) were measured as described.

Discussion

In order to obtain a better understanding of how the components of the Ras/cAMP pathway regulate growth and cell proliferation, we screened for targets of the protein kinase Rim15, a kinase previously shown to control a subset of growth and cell cycle-related targets of PKA. In this context, we performed a dosage suppressor screen for genes that, when overexpressed, rescued the defect of *rim15Δ* mutants to derepress *SSA3-lacZ* upon nutrient limitation. It had been shown that Rim15 is required for proper entry into stationary phase (Reinders *et al.*, 1998), antagonizing PKA-mediated proliferative growth. Therefore, downstream targets of Rim15 may likely include growth inhibitory gene products. In a screen for dosage suppressors of *rim15Δ*, overexpression of genes whose products act inhibitory for growth may either not grow, or have a selective disadvantage compared to the majority of the transformed gene pool. To overcome this caveat, we used a conditional, *GAL1*-inducible expression library, which consists of independent recombinants of yeast genome fragments that represent a

breakpoint at every base in the yeast genome (Ramer *et al.*, 1992). This system allowed us to grow the transformants under non-inducing conditions on glucose, circumventing the limitation of standard high-copy-number libraries which may not allow isolation of growth inhibitory genes.

The initial screen yielded an unexpected high variety in gene products with various biochemical functions and, surprisingly, almost no redundancy in the identified clones, which indicates that the screen was far from being saturated. Since the number of independently screened clones (i.e. 2.5×10^6) predicts that each gene may be found at least 100 times, it is likely that the number of independent clones may have significantly dropped during amplification of the library via *E. coli*. In addition, we found that many of the positive yeast clones carried library plasmids without inserts, indicating that the observed suppression may be due to genetic alterations in the genome. This assumption is supported by the finding that a high number of isolated plasmids (i.e. 27) was not able to complement the *rim15Δ* phenotype upon retransformation. Thus, the relative ease with which genomic suppressors may be picked up greatly enhances the background and makes it relatively difficult to isolate true positive clones.

Nevertheless, inclusion of further selection criteria, such as ability to suppress the *rim15Δ* mutant defect in sporulation and in proper G_1 arrest upon nutrient limitation, allowed us to isolate 6 true positive plasmid clones. When expressed from the strong inducible *GAL1* promoter, these 6 clones also exacerbated the temperature-sensitive growth of a *tpk1 tpk2^{ts} tpk3* strain. These results suggest that the products of these 6 genes may either act downstream of, or in parallel to the Ras/cAMP pathway to negatively regulate growth.

Two of the isolated genes, *YNL335W* and *YPL041C*, encode proteins of yet unknown function, and it will be of particular interest to elucidate their putative interaction with the Ras/cAMP pathway. Is noteworthy to mention in this context that there exists a second open reading frame in yeast, *YFL061W*, that shares 100% identity to *YNL335W* in its deduced amino acid sequence. It is therefore likely that phenotypes caused by loss of these proteins may have escaped in systematic genome-wide analysis projects carried out nowadays, and further analysis will consequently have to include both genes.

A third gene identified codes for the protein kinase Pkc1. Pkc1 is involved in regulation of cell wall integrity in yeast. The yeast cell wall must be remodeled in response to environmental cues, as well as in response to cell cycle specific changes such as budding and

mating. Pkc1 is activated by the Rho-like GTPase Rho1 and acts as an activator of a MAP kinase cascade to regulate downstream targets (Cid *et al.*, 1995; Heinisch *et al.*, 1999). Although it seems likely that this pathway acts in parallel to the Ras/cAMP pathway, multiple connections to other pathways have emerged in the past few years, and it will be of interest to elucidate whether there may also be a cross talk between the Ras/cAMP pathway and Pkc1 involving Rim15, since it is likely that entry into stationary phase is associated with alterations in the cell wall as well.

A fourth gene encodes Cdh1, a component of the anaphase promoting complex (APC). The anaphase promoting complex is a multisubunit ubiquitin-protein ligase, that is required for proteolysis of mitotic cyclins (Visintin *et al.*, 1997). Proteolysis mediated by the APC triggers chromosome segregation and exit from mitosis, and plays therefore a key role in cell cycle control. Previous reports suggested that the Ras/cAMP pathway may interact with and control the activity of the APC, in order to control progression through mitosis (Anghileri *et al.*, 1999; Irniger *et al.*, 2000). Interestingly, it has been shown that proteolysis of mitotic cyclins in yeast required association of the APC with the substrate-specific activator Cdh1, and that phosphorylation of Cdh1 by CDKs blocked the Cdh1-APC interaction (Zachariae *et al.*, 1998). An involvement of protein kinase Rim15 in regulation of APC activity via Cdh1 would provide a direct link between the Ras/cAMP pathway and the cell cycle control machinery.

The last two genes code for putative transcription factors. One of these, namely Tye7, has been shown to function as transcriptional activator that induces expression of glycolytic genes in a parallel pathway to Gcr1 (Sato *et al.*, 1999). This is rather surprising, since glycolysis is shut off by the main glucose repression pathway in the post-diauxic phase. How a transcriptional activator of glycolytic genes may suppress the loss of Rim15 remains to be explained. The other putative transcription factor, namely Gis1, encodes a zinc-finger protein previously reported to be involved in DNA repair (Balciunas and Ronne, 1999). Preliminary studies of epistasis indicated that it may interact with the Ras/cAMP pathway. Therefore we gave preference to studies on the role of Gis1 as potential downstream target of the Ras/cAMP pathway as illustrated in the following.

Chapter III

Saccharomyces cerevisiae Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1

Parts of this chapter have been published in:

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The *Saccharomyces cerevisiae* protein kinase Rim15 was identified previously as a component of the Ras/cAMP pathway acting immediately downstream of cAMP-dependent protein kinase (PKA) to control a broad range of adaptations in response to nutrient limitation. Here, we show that the zinc finger protein Gis1 acts as a dosage-dependent suppressor of the *rim15Δ* defect in nutrient limitation-induced transcriptional derepression of *SSA3*. Loss of Gis1 results in a defect in transcriptional derepression upon nutrient limitation of various genes that are negatively regulated by the Ras/cAMP pathway (*e.g., SSA3, HSP12*, and *HSP26)*. Tests of epistasis as well as transcriptional analyses of Gis1-dependent expression indicate that Gis1 acts in this pathway downstream of Rim15 to mediate transcription from the previously identified post-diauxic shift (PDS) element. Accordingly, deletion of *GIS1* partially suppresses, and overexpression of *GIS1* exacerbates the growth defect of mutant cells that are compromised for PKA activity. Moreover, PDS element-driven expression, which is negatively regulated by the Ras/cAMP pathway and which is induced upon nutrient limitation, is almost entirely dependent on the presence of Gis1.

Saccharomyces cerevisiae Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1

Introduction

The yeast *Saccharomyces cerevisiae* copes with diverse environmental conditions by coordinated regulation of growth, cell cycle progression, and metabolic activities. The Ras/cAMP pathway is essential for the control and integration of these processes, in particular with respect to the nutritional status. The dramatic reprogramming of the metabolism at the diauxic transition, when glucose becomes limiting, as well as some of the subsequent adaptations during both the post-diauxic phase, when cells grow respiratively on ethanol, and during entry into stationary phase, are negatively controlled by the Ras/cAMP pathway. Thus, cells with elevated PKA activity fail to accumulate carbohydrate reserves, to undergo a proper diauxic transition, and to arrest in G_1 upon nutrient limitation. As a result, such cells remain highly sensitive to heat stress and rapidly lose viability in stationary phase. In contrast, PKA-deficient cells exhibit physiological changes normally associated with nutrient limitation such as G_1 cell-cycle arrest, accumulation of storage carbohydrates, and increased resistance towards heat and oxidative stress (for reviews and further details on the Ras/cAMP pathway see Tatchell, 1986; Broach and Deschenes, 1990; for a review on stationary phase see Werner-Washburne *et al.*, 1993; Thevelein and de Winde, 1999).

The mechanism by which PKA controls growth is still an issue of conjecture. One mechanism by which cells may control proliferation has recently been suggested to be based on PKA-mediated regulation of G_1 cyclin translation (Hall *et al.*, 1998). In addition, it was found that modulation of Rap1 transcriptional activity by PKA mediates growth-regulated expression of ribosomal protein genes (Klein and Struhl, 1994). However, positive PKA-mediated regulation of ribosomal protein synthesis may not be the only reason for the dependence of growth on PKA (Klein and Struhl, 1994; Neuman-Silberberg *et al.*, 1995) and some of the growth-related effects of PKA may be due to changes in additional transcriptional control mechanisms. For instance, transcriptional derepression/activation of a large number of yeast genes (*e.g.*, *CTT1*, *DDR1*, *HSP12*, and *TPS2*) is negatively regulated by PKA through one or more AG_4 stress-responsive elements (STREs), which confer transcriptional activation in response to a wide range of stresses, including heat, oxidative and osmotic shocks, and nutrient limitation (for reviews see Mager and De Kruijff, 1995; Ruis and Schuller, 1995). Control of STRE-driven gene expression by PKA is mediated via Msn2 and Msn4, a pair of

partially redundant zinc finger transcription factors that recognize and bind to STRE (Estruch and Carlson, 1993; Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996; Gorner *et al.*, 1998). Surprisingly, loss of Msn2 and Msn4 renders cells largely independent of PKA activity, suggesting that an essential function of PKA is to inhibit expression of growth-inhibitory genes which are under control of Msn2 and Msn4 (Smith *et al.*, 1998). In agreement with this suggestion, Msn2/Msn4 function is also required for expression of *YAK1*, a gene whose product was previously shown to antagonize PKA-dependent growth (Garrett and Broach, 1989; Garrett *et al.*, 1991; Smith *et al.*, 1998). PKA-mediated control of gene expression is also exerted via additional transcriptional regulators. Accordingly, expression of the glucose-repressible *ADH2* gene has been found to be mainly regulated by PKA-dependent inactivation of the transcriptional activator Adr1 (Cherry *et al.*, 1989), while expression of *GAC1* and *SSA3* was reported to be possibly regulated by PKA-dependent activation of the transcriptional repressor Sok2 (Ward *et al.*, 1995). Since overexpression of *SOK2* can partially relieve dependence on PKA function, Sok2 acts as a PKA-dependent repressor of one or more genes whose products inhibit cell proliferation.

Several observations suggest the existence of additional PKA-dependent transcriptional control mechanisms. For instance, expression of *GSY2* was recently found to be partially repressed by the PKA pathway through an unknown STRE-independent mechanism (Parrou *et al.*, 1999). Similarly, a large-scale analysis of gene expression in a *msn2 msn4* double mutant (Boy-Marcotte *et al.*, 1998), as well as a screen for cAMP-repressible genes (Tadi *et al.*, 1999), revealed a subset of Msn2/Msn4-independent genes whose expression is negatively controlled by cAMP. One PKA-regulated element which may confer Msn2/Msn4-independent transcriptional control is the T(T/A)AG_3AT post-diauxic shift (PDS) element which mediates transcriptional activation in response to nutritional limitation (Boorstein and Craig, 1990). Unlike the very similar STRE, it does not mediate transcriptional activation in response to other stresses such as heat shock (Boorstein and Craig, 1990), indicating that STRE and PDS elements may be controlled by different PKA-dependent mechanisms. In agreement with such a model, Msn2 and Msn4 were found to be dispensable for the nutrient limitation-induced transcriptional activation of *SSA3*, a gene with several PDS elements, but no conserved STRE in its promoter region (Martinez-Pastor *et al.*, 1996). In this context, it is interesting to note that transcriptional derepression/activation of *SSA3* at the diauxic transition is almost entirely dependent on the presence of the protein kinase Rim15, which has been identified as a component of the Ras/cAMP pathway acting immediately downstream and

under negative control of PKA to control a broad range of adaptations in response to nutrient limitation (Reinders *et al.*, 1998).

Here we describe the identification of *GIS1* as a dosage-dependent suppressor of the *rim15Δ* defect to derepress/activate *SSA3* upon nutrient limitation. *GIS1* encodes a zinc finger transcription factor which has recently been isolated as a damage-responsive repressor of the photolyase-encoding *PHR1* gene (Jang *et al.*, 1999), as well as a high-copy suppressor of *gig1-2*, a mutation in the *SRB8* gene coding for a subunit of the cyclin C-dependent protein kinase complex (Balciunas and Ronne, 1999). Loss of Gis1 results in a defective response of mutant cells to nutrient limitation, including a defect in transcriptional derepression/activation of *SSA3*. Tests of epistasis as well as transcriptional analyses of Gis1-dependent expression suggest that Gis1 acts in the Ras/cAMP pathway downstream of Rim15 to specifically control transcriptional activation through the PDS element. Interestingly, overexpression of *GIS1* prevents growth and loss of Gis1 partially relieves the dependence on PKA function, indicating that Gis1 activates one or more genes whose products inhibit cell proliferation

Results

Identification of *GIS1* as dosage suppressor of *rim15Δ*

We have previously reported that loss of Rim15 results in a defective response to nutrient limitation: mutant cells lacking Rim15 fail to accumulate trehalose, to derepress *HSP12, HSP26*, and *SSA3*, to induce thermotolerance, and to properly arrest in G_1 (Reinders *et al.*, 1998). To identify potential downstream effectors of Rim15, we performed a screen for dosage-dependent suppressors of the *rim15Δ* defect in nutrient limitation-induced transcriptional derepression of *SSA3*. Accordingly, strain AR2 (*rim15Δ/rim15Δ)* containing the reporter plasmid pWB204Δ-236 (SSA3-lacZ, Boorstein and Craig, 1990) was transformed with a genomic DNA library under the control of the strong, inducible *GAL1* promoter (Ramer *et al.*, 1992). We rescued library plasmids from cells in which the reporter gene showed galactose-dependent transcription after replica-plating and incubation for 2 days on selective X-Gal plates (containing 2% galactose and 1% raffinose) and assigned the plasmids to 48 different classes by restriction analysis. To specifically isolate downstream effectors of the Ras/cAMP/Rim15 pathway, we further tested the positive plasmids for their ability to exacerbate the growth defect of a strain with attenuated PKA activity. To this end, one

representative plasmid of each class was assayed for galactose-dependent inhibition of growth at 31.5°C of a *tpk2*^{ts} strain (SGY446). Partial sequencing of a plasmid from one of the 6 remaining positive classes showed it to contain the entire *GIS1* open reading frame, including 470 bp of the 5' untranslated region, fused to the *GAL1* promoter of the library plasmid. *GIS1* encodes a 90-kDa protein which contains one classical C_2H_2 zinc finger followed by an alternative C_2HC zinc finger at the carboxy-terminal end (Bohm *et al.*, 1997). Comparison of the predicted Gis1 sequence to all other deduced amino acid sequences in yeast revealed a second gene product, Rph1, with particularly high homology to the zinc finger containing carboxy-terminal region of Gis1 (Balciunas and Ronne, 1999; Jang *et al.*, 1999). Interestingly, while genetic studies suggested different roles for Gis1 and Rph1 (Balciunas and Ronne, 1999), *in vivo* footprinting, binding competition, and transcriptional studies indicated that Gis1 and Rph1 may act as functionally redundant transcriptional repressors of a STRE-like upstream repressing sequence (URSPHR1, Jang *et al.*, 1999).

Gis1 regulates nutrient limitation-induced transcription of several PKA/Rim15-dependent genes

To determine the consequences of the loss of Gis1, we replaced the complete *GIS1* coding region by a PCR-based gene deletion method (Baudin *et al.*, 1993; Wach *et al.*, 1994, see Materials and methods). In agreement with previous studies (Balciunas and Ronne, 1999; Jang *et al.*, 1999), deletion of *GIS1* caused no obvious defect in germination of spores, or in exponential growth on YPD at 30°C. Thus, *GIS1* is not essential for growth or germination. The effect of *gis1Δ* on *SSA3* transcription was assessed by using the *SSA3-lacZ* reporter plasmid pWB204Δ-236. Comparison with wild-type cells revealed that *gis1Δ* cells, similarly to *rim15Δ* cells, were strongly defective for nutrient limitation-induced derepression of *SSA3-lacZ* (Table I). Overexpression of *GIS1*, in contrast, was not only able to fully suppress the *SSA3-lacZ* derepression defect of *rim15Δ*cells, but also to significantly increase *SSA3-lacZ* induction in wild-type, *rim15Δ*, and *gis1Δ* mutant cells when compared to the control plasmid-containing wild-type strain. Finally, the increase in *SSA3-lacZ* induction mediated by *RIM15* overexpression was found to be almost entirely dependent on the presence of Gis1 (Table I). The simplest interpretation of these results is that Gis1 may act downstream of Rim15 to mediate one or more Rim15-controlled, nutrient limitation-induced responses. Therefore, we also analyzed whether deletion of *GIS1* causes additional defects in response to

Table I. Epistatic relationship between *RIM15* and *GIS1*

Plasmid	Strain		
	Wild type	*rim15Δ*	*gis1Δ*
YCpIF2	153.0 ± 23.1	33.3 ± 9.0	22.1 ± 4.0
YCpIF2-*RIM15*	280.9 ± 54.6	249.6 ± 58.5	34.1 ± 2.9
YCpIF2-*GIS1*	264.5 ± 45.8	281.5 ± 54.4	258.7 ± 36.3

Transformants of wild-type (AR1-1A), *rim15Δ* (AR1-1C), and *gis1Δ* (CDV100-10D) cells were grown to early stationary phase (2 days) on SD medium containing 2% galactose and 1% raffinose to induce *GAL1*-driven transcription of *RIM15* and *GIS1* in the corresponding YCpIF2-derived plasmids. β-Galactosidase activities were measured (as described in Miller, 1972) to monitor the induction of an *SSA3-lacZ* fusion gene (from plasmid pWB204Δ-236). Notably, all YCpIF2-*GIS1* transformants reached much lower cell densities (< 30%) than corresponding YCpIF2 and YCpIF2-*RIM15* transformants. Values represent means ± S.D.s of β-galactosidase activities (in Miller units) of three independent experiments.

nutrient limitation. As is seen in Figure 1, *SSA3*, *HSP12*, and *HSP26* were repressed in exponentially growing wild-type cells and induced upon glucose starvation. Under the same conditions, *gis1Δ* mutant cells, similar to *rim15Δ* and *gis1Δ rim15Δ* double mutant cells, were almost entirely defective for derepression of *SSA3* and partially defective for the maintenance of derepression of *HSP12* (Figure 1; lane 4). Derepression of *HSP26*, in contrast, was found to be only slightly defective in *gis1Δ* mutant cells, while being strongly defective in *rim15Δ* and *rim15Δgis1Δ* double mutant cells (Figure 1; lanes 3 and 4). Notably, glucose limitation-induced *TPS2* transcription remained largely unaffected by *gis1Δ* and/or *rim15Δ*. As a control for the physiological status of the cells, we also examined the expression pattern of a gene, *SSB1,* the transcription of which is repressed upon nutrient limitation (Werner-Washburne *et al.*, 1989). Transcript levels of *SSB1* were high in exponentially growing cells and very low in glucose-starved cells of all four strains (Figure 1; lanes 1-4). Together, these results show that Gis1 is not only involved in nutrient limitation-induced derepression of *SSA3*, but also

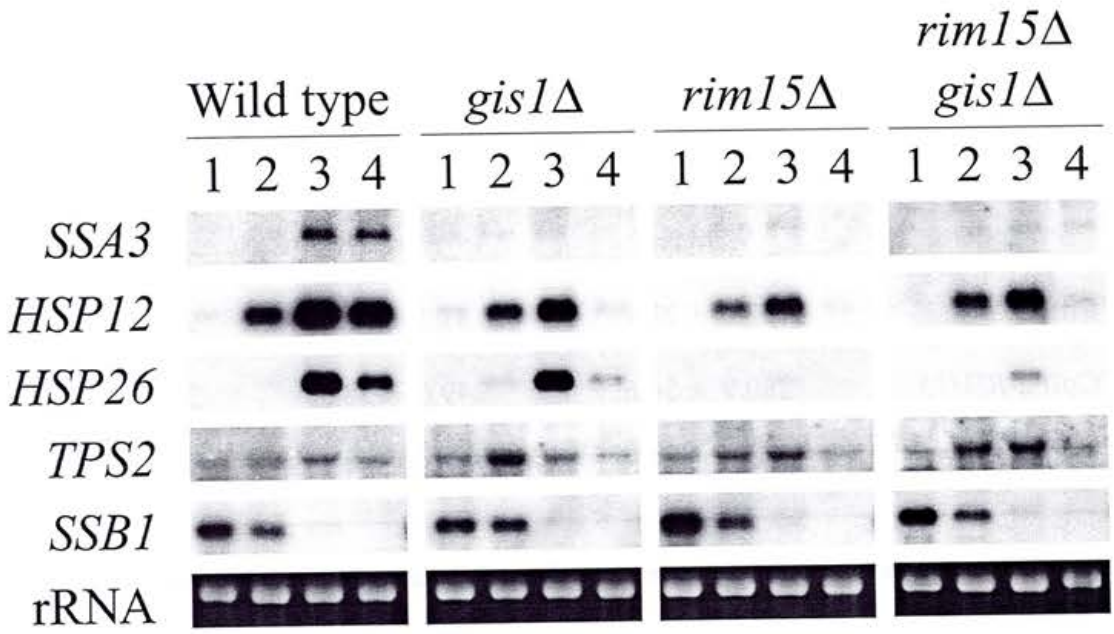

Fig. 1 Effects of *gis1Δ*, *rim15Δ*, and *gis1Δ rim15Δ* on the expression of various genes following glucose starvation. Homozygous wild-type (YEF473), and *gis1Δ/gis1Δ* (CDV101), *rim15Δ/rim15Δ* (AR2), and *gis1Δ/gis1Δ rim15Δ/rim15Δ* (CDV104) mutant cells were harvested by centrifugation and resuspended in S medium containing 0.1% glucose. Total RNAs were extracted immediately after resuspension (1) and following further incubation for 90 min (2), 180 min (3), and 270 min (4). Equal amounts of RNAs (10 μg) were probed with *SSA3, HSP12, HSP26, TPS2*, and *SSB1* fragments after electrophoresis and blotting. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining.

partially regulates expression of additional PKA-repressible genes such as *HSP12* and *HSP26* (Engelberg *et al.*, 1994; Varela *et al.*, 1995).

The strong defect in *HSP26* derepression observed in *rim15Δ*cells is only partially mimicked by loss of Gis1, indicating that Gis1 mediates only a subset of Rim15-controlled responses. Therefore, diploid *gis1Δ/gis1Δ* cells were also analyzed for a variety of other phenotypic traits which are characteristic of *rim15Δ* cells (e.g., the defects in trehalose accumulation, in proper G1 arrest, in long term stationary phase survival, as well as in sporulation, Vidan and Mitchell, 1997; Reinders *et al.*, 1998). When compared to isogenic wild-type cells (YEF473), 4-days old stationary phase *gis1Δ/gis1Δ* cells (CDV101) exhibited normal trehalose levels (0.207 ± 0.029 g/g protein versus 0.176 ± 0.008 g/g protein for *GIS1/GIS1* cells) and were only slightly defective for proper G_1 arrest (3.5 ± 0.4% budded cells versus 0.8 ± 0.1% budded cells for *GIS1/GIS1* cells). In addition, *gis1Δ/gis1Δ* cells exhibited no obvious

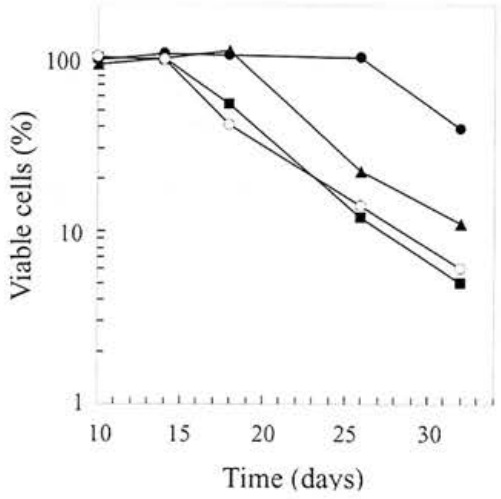

Fig. 2 Effects of *gis1Δ*, *rim15Δ*, and *gis1Δ rim15Δ* on stationary phase survival. Homozygous wild-type (YEF473; closed circles), and *gis1Δ/gis1Δ* (CDV101; closed triangles), *rim15Δ/rim15Δ* (AR2; open circles), and *gis1Δ/gis1Δ rim15Δ/rim15Δ* (CDV104; closed squares) mutant cells were grown to stationary phase on YPD medium. The percentage of viable cells was determined by the colony-forming efficiency on YPD agar at the times indicated.

sporulation defect (data not shown). However, *gis1Δ/gis1Δ* cells were found to be significantly more sensitive than wild-type cells, and only slightly less sensitive than *rim15Δ* and *rim15Δ gis1Δ* cells, to prolonged nutrient starvation (Figure 2). These results suggest that Gis1 controls transcriptional activation of a subset of Rim15-dependent genes, the expression of which is important for long term survival under conditions of nutrient starvation. In agreement with such a role for Gis1, we found Gis1 protein levels to be very low in exponentially growing cells, as estimated by immune blots of myc13-tagged Gis1 (lane 1; Figure 3), and to increase following glucose exhaustion at the diauxic transition (lanes 2-5; Figure 3). Interestingly, Gis1 protein levels were similarly induced in *rim15Δ* cells entering the diauxic transition (Figure 3), indicating that potential Rim15-dependent regulation of Gis1 activity is exerted at a post-transcriptional level.

Loss of Gis1 partially suppresses *cdc25^{ts}*, *cdc35^{ts}*, and *tpk^{ts}*

The data presented above suggest that Gis1 may act in the Ras/cAMP pathway downstream of Rim15 to control transcriptional activation upon nutrient limitation. To determine the potential contribution of Gis1 to PKA-dependent growth, we tested whether loss of Gis1

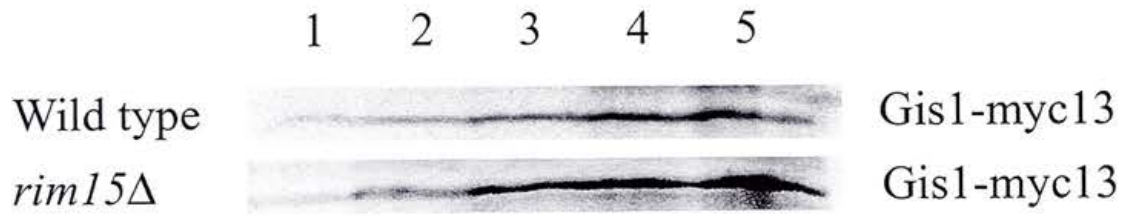

Fig. 3 Immunoblot analysis of Gis1-myc13 in wild-type (NB14) and *rim15Δ* mutant (NB15) cells entering the diauxic transition phase. Samples were taken 2 h before glucose exhaustion (1), at the time of glucose exhaustion (2), and 2 h (3), 4 h (4), and 6 h (5) following glucose exhaustion. Twenty micrograms of extract protein were loaded in each lane.

could suppress growth defects that are associated with attenuated PKA activity. To this end, temperature-sensitive Ras GTP-exchange factor and adenylate cyclase mutant strains, harboring corresponding *cdc25^{ts}* and *cdc35^{ts}* mutations, respectively, as well as a temperature-sensitive PKA mutant strain with only one functional *tpk2^{ts}* gene, were transformed with the *gis1Δ::kanMX2* cassette. In all three mutant strains, deletion of *GIS1* did not restore growth at the non-permissive temperature, indicating that *gis1Δ* is not able to suppress the total loss of PKA activity (data not shown). However, when tested at a semi-permissive temperature (*i.e.* at 34°C), the relatively low growth rates of each of the three temperature-sensitive mutant strains were significantly increased by loss of Gis1. Accordingly, deletion of *GIS1* caused growth rates of *cdc25^{ts}*, *cdc35^{ts}*, and *tpk2^{ts}* cells to increase from 0.158 ± 0.008 h^{-1} (OL86) to 0.205 ± 0.006 h^{-1} (NB19), from 0.147 ± 0.007 (PD6517) to 0.181 ± 0.008 h^{-1} (NB21), and from 0.151 ± 0.005 h^{-1} (SGY446) to 0.191 ± 0.002 h^{-1} (NB23), respectively. Together, these results show that loss of Gis1 partially relieves dependence on PKA function.

GIS1 overproduction exacerbates the growth defect of *cdc25^{ts}*, *cdc35^{ts}*, and *tpk^{ts}* strains

In order to determine whether Gis1, as suggested by the results presented above, may antagonize PKA-dependent growth, we tested whether *GIS1* overexpression could exacerbate the growth defect associated with attenuated PKA activity. Overexpression of *GIS1* from the galactose-inducible *GAL1* promoter inhibited growth even in wild-type cells (Figure 4A; see also Table I). In contrast, wild-type cells overexpressing *GIS1* from the constitutive *ADH1* promoter had only a slight growth defect which was apparent after growth for one day on

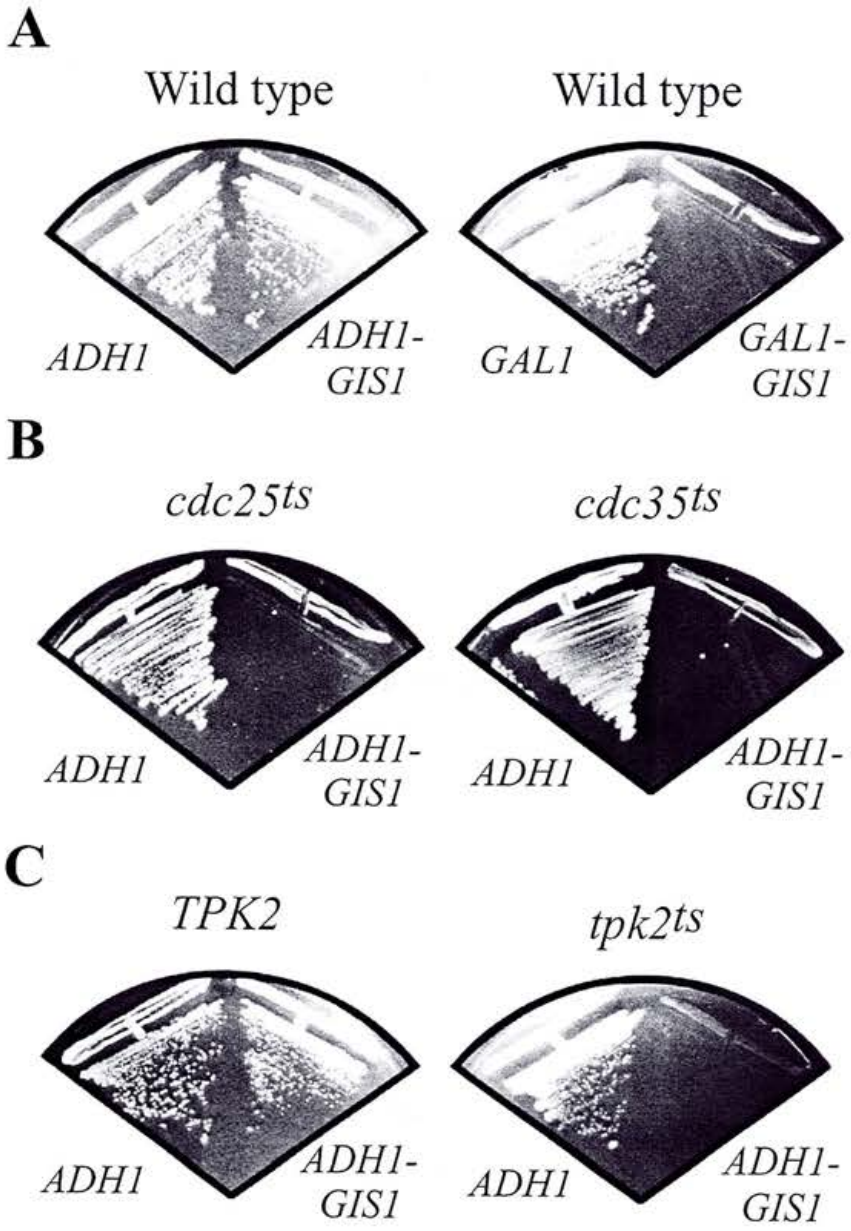

Fig. 4 *GIS1* overproduction inhibits growth of wild-type cells and exacerbates the growth defect of *cdc25*^{ts}, *cdc35*^{ts}, and *tpk2*^{ts} strains. (A) Wild-type strain YEF473 was transformed with plasmids YCp*ADH1* (*ADH1*), YCp*ADH1-GIS1* (*ADH1-GIS1*), YCpIF2 (*GAL1*), and YCpIF2-*GIS1* (*GAL1-GIS1*), streaked on glucose- (*ADH1* plasmids), or galactose-containing (*GAL1* plasmids) selective plates, and incubated for three days at 30°C. (B) Strains OL86 (*cdc25*^{ts}) and PD6517 (*cdc35*^{ts}) were transformed with plasmids YCp*ADH1* and YCp*ADH1-GIS1*, streaked on glucose-containing SD plates, and incubated for three days at 27°C. (C) Strain SGY446 (*tpk2*^{ts}) and its isogenic wild-type strain ASY18 (*TPK2*) were transformed with plasmids YCp*ADH1* and YCp*ADH1-GIS1*, streaked on glucose-containing SD plates, and incubated for three days at 27°C.

plates (data not shown), but not when plates were incubated for more than 2 days (Figure 4A). In accordance with this result, assessment of growth in liquid cultures revealed that the growth rate of *ADH1-GIS1* overexpressing wild-type cells was reduced by approximately 50% in comparison to control cells (data not shown). When transformed into $cdc25^{ts}$ and $cdc35^{ts}$ mutants, the *GIS1* overexpression plasmid YCp*ADH1-GIS1*, but not the corresponding control plasmid YCp*ADH1*, seriously compromised both mutants for growth even at the permissive temperature of 27°C (Figure 4B). Similarly, overexpression of *GIS1* from the *ADH1* promoter inhibited growth of $tpk2^{ts}$ cells at the permissive temperature of 27°C, while it had little effect on the growth of isogenic *TPK2* wild-type cells. Thus, Gis1 overproduction partially inhibits growth in wild-type cells and exacerbates the growth defects of PKA-compromised mutants.

To study further the effects of *GIS1* overexpression in wild-type cells, and to compare these effects to overexpression of *RIM15* and the *GIS1* homolog *RPH1*, strain YEF473 was transformed with YCp*ADH1*, YCp*ADH1-RIM15*, YCp*ADH1-GIS1*, and YCp*ADH1-RPH1*

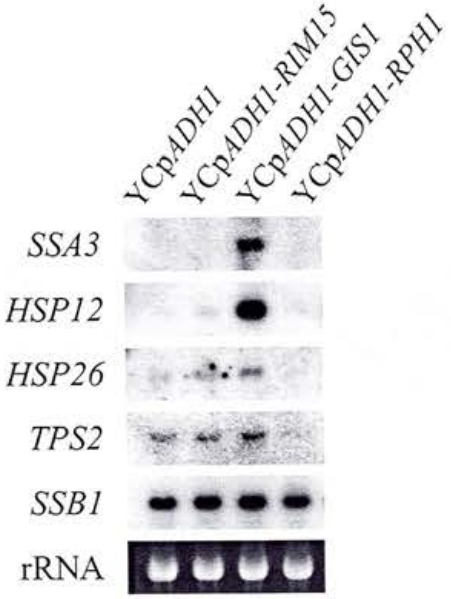

Fig. 5 Overexpression of *GIS1*, but not of *RPH1*, induces transcriptional activation of *SSA3, HSP12,* and *HSP26*. Wild-type (YEF473) cells transformed with either YCp*ADH1*, YCp*ADH1-RIM15*, YCp*ADH1-GIS1*, or YCp*ADH1-RPH1* were grown to exponential phase on SD media containing 2 % glucose. Total RNAs from exponentially growing transformants were extracted and equal amounts of RNAs (10 µg) were probed with *SSA3, HSP12, HSP26, TPS2*, and *SSB1* fragments after electrophoresis and blotting. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining.

and analyzed for transcription of *SSA3, HSP12, HSP26, TPS2* and *SSB1* in exponentially growing cells. Transcript levels of *SSA3, HSP12*, and *HSP26* were barely detectable in wild-type cells growing exponentially on glucose-containing medium, but were strongly induced following overexpression of *GIS1* under the same conditions (Figure 5; lanes 1 and 3). Similarly, albeit to a significantly lower extent, overexpression of *RIM15* was found to cause transcriptional induction of *HSP12* and *HSP26*, while transcription of the same genes was not induced by overexpression of *RPH1* (Figure 5; lanes 1, 2, and 4). In contrast, the level of *TPS2* expression detected in exponentially growing cells remained largely unaffected by *GIS1* and *RIM15* overexpression, but was significantly reduced by overexpression of *RPH1*, suggesting that Rph1 may function as a transcriptional repressor of *TPS2* under these conditions (Figure 5; lane 4). Together, these results show that overexpression of *GIS1* allows derepression/activation of PKA-repressible genes even under repressing conditions.

Transcriptional activation of the PDS element, but not of STRE, is dependent on Gis1

The data presented above suggest that Gis1 functions as a transcriptional activator of PDS element- and/or STRE-controlled genes. Therefore, we next determined whether Gis1 acts as an activator of transcription using the one-hybrid technique. To this end, we fused the entire Gis1 to the LexA DNA-binding domain (DBD) in plasmid pEG202 (see Materials and methods). This fusion protein was able to strongly activate transcription from a promoter containing 8 *lexA*-binding sites (Table II). This is in agreement with a previously reported observation that a partial Gis1 protein, lacking the carboxy-terminal zinc finger domain, can activate transcription in a one-hybrid assay (Balciunas and Ronne, 1999). Interestingly, we were not able to detect transcriptional activity of a corresponding LexA-Rph1 fusion protein when assayed under the same conditions. Moreover, transcriptional activity of Gis1, measured by the one-hybrid assay, was equally strong in exponentially growing as in stationary phase wild-type cells, but slightly reduced in corresponding *rim15Δ* cells (Table II). These results show that Gis1 can act as a transcriptional activator that, under the conditions of the one-hybrid assay, is unresponsive to the nutritional status and only partially dependent on the presence of Rim15. These results may be explained if overexpression of *GIS1* from the strong *ADH1* promoter in pEG202 results in protein levels that exceed a certain threshold beyond which Gis1 becomes largely independent of activation by upstream regulatory proteins (see Discussion).

Table II. Transcriptional activation by LexA-Gis1

Expressed proteins	Wild type		*rim15Δ*	
	LOG	STAT	LOG	STAT
LexA	1.1 ± 0.1	7.1 ± 2.8	5.1 ± 0.1	1.6 ± 0.3
LexA-Gis1	1268.4 ± 65.6	1157.0 ± 167.1	981.9 ± 256.7	922.3 ± 25.0
LexA-Rph1	9.5 ± 2.5	11.5 ± 5.0	25.2 ± 2.9	25.0 ± 5.1

Proteins were expressed from plasmids pEG202-*GIS1* and pEG202-*RPH1* and from vector pEG202 (Zervos *et al.*, 1993). Target plasmid was pSH18-34 containing 8 *LexA* operators (Gyuris *et al.*, 1993). Transformants of wild-type (AR1-1A) and *rim15Δ* (AR1-1C) cells were grown with selection for both plasmids. β-Galactosidase activities were measured (as described in Miller, 1972) to monitor the induction of the *LexAops-lacZ* fusion gene (from plasmid pSH18-34). Values represent means ± S.D.s of β-galactosidase activities (in Miller units) of three independent transformants.

Gis1-dependent transcriptional activation may be mediated via STREs and/or PDS elements. To quantitatively assess the role of Gis1 for STRE- and PDS element-driven expression, nutrient limitation-induced expression of heterologous *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ* genes were tested in different strain backgrounds. As expected, nutrient limitation significantly induced both STRE- and PDS element-driven expression in wild-type cells (Figure 6A and B). The induction ratio (defined as the ratio of nutrient limitation-induced expression to basal level expression) of the *STRE-LEU2-lacZ* reporter was slightly reduced in *rim15Δ* cells (26.8% decrease), remained unchanged in *gis1Δ* and *rph1Δ* cells, and was strongly reduced in both *msn2 msn4* (89.8% decrease) and *msn2 msn4 gis1Δ* (94.1% decease) cells when compared to the induction ratio in wild-type cells (Figure 6A). These results show that STRE-driven expression, while being strongly dependent on the presence of Msn2 and Msn4, is completely independent of Gis1 and Rph1, and only partially dependent on Rim15. The induction ratio of the *PDS-LEU2-lacZ* reporter, in contrast, was strongly reduced in *rim15Δ* (85.1% decrease), *gis1Δ* (96.5% decrease), and *msn2 msn4 gis1Δ* (95.0% decrease) cells, while it remained virtually unchanged in *msn2 msn4* and *rph1Δ* cells when compared to

the induction ratio in wild-type cells (Figure 6B). Thus, PDS element-driven expression is completely independent of Msn2, Msn4, and Rph1, strongly dependent on Rim15, and almost entirely dependent on the transcriptional activator Gis1.

Finally, STRE-driven basal-level expression has previously been reported to be enhanced when cells were growing on rich medium containing ethanol instead of glucose as carbon

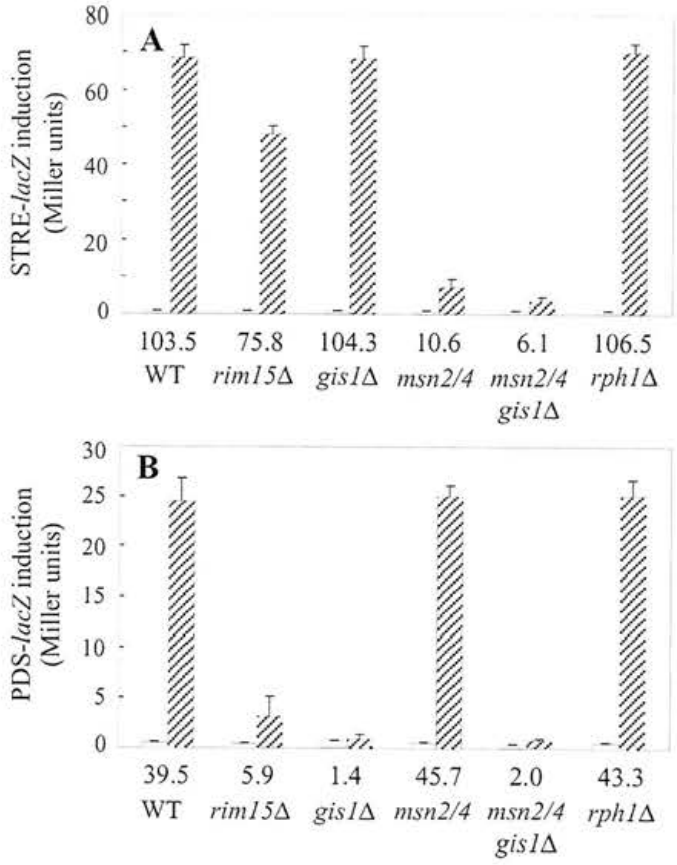

Fig. 6 Effects of *rim15Δ*, *gis1Δ*, *msn2 msn4*, *msn2 msn4 gis1Δ*, and *rph1Δ* mutations on the basal-level expression and nutrient limitation-induced expression of STRE- and PDS element- driven *LEU2-lacZ* reporter genes. Wild type, *rim15Δ*, *gis1Δ*, *msn2 msn4*, *msn2 msn4 gis1Δ*, and *rph1Δ* cells carrying a single chromosomally integrated STRE-*LEU2-lacZ* reporter gene (strains CDV120, CDV121, CDV122, CDV123, CDV124, and IP17, respectively) (A), or a corresponding PDS-*LEU2-lacZ* reporter gene (strains CDV125, CDV126, CDV127, CDV128, CDV129, and IP13, respectively) (B), were grown to exponential (open bars) and to early stationary phase (2 days; cross-hatched bars) on YPD medium. The chromosomal genotypes are indicated below the ordinate; the induction ratios following nutrient limitation are indicated above the chromosomal genotypes. β-Galactosidase activities were measured (as described in Miller 1972) to monitor the induction of the STRE-*LEU2-lacZ and* PDS-*LEU2-lacZ* fusion genes. Values represent means ± S.D.s of β-galactosidase activities (in Miller units) of three independent experiments.

source (Martinez-Pastor *et al.*, 1996). In this context, we found that expression from both the heterologous *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ* genes, while being very low in wild-type cells growing exponentially on glucose (0.5 ± 0.1 and 0.6 ± 0.1 Miller units for *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ*, respectively), was significantly enhanced in cells growing exponentially on ethanol (30.0 ± 5.1 and 15.3 ± 3.8 Miller units for *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ*, respectively), or glycerol (46.8 ± 15.3 and 25.0 ± 7.8 Miller units for *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ*, respectively). Thus, both STRE- and PDS element-driven expression are repressed by the presence of glucose.

2D-gel analyses of *rim15Δ* and *gis1Δ* strains reveal new PDS element-controlled genes

To identify genes that are under control of Rim15 and Gis1, we were able to use the techniques of two dimensional (2D) gel-electrophoresis with coupled analysis by mass spectrometry in a collaboration with Dr. H. Voshol (Novartis, Basel). Wild-type (YEF473), *rim15Δ* (AR2) and *gis1Δ* (CDV101) cells were grown to diauxic shift phase and whole cell extracts were subjected to 2D gel electrophoresis. The expression pattern on the three gels were compared and spots that show a distinguishable difference in expression in the two mutant strains when compared to the wild-type strain were chosen. The corresponding peptides were identified by MALDI/MS. We were provided with a short summary of identified genes as depicted in Fig. 7. As expected, the presented dataset included genes that are already known to be dependent on Rim15 and Gis1 for expression, like *HSP12* and *HSP26*, confirming the quality of the applied technique. Additionally, new genes were identified that showed a similar expression pattern like *HSP12* and *HSP26*. Accordingly, transcriptional derepression of *YPL223C* during the post diauxic phase was strongly dependent on Rim15 and Gis1 as its expression is nearly absent in the *rim15Δ* and *gis1Δ* mutant strains. Analysis of the *YPL223C/GRE1* ORF revealed that it contains six PDS elements in its promoter region (Garay-Arroyo and Covarrubias, 1999). To verify the effect of loss of Rim15 and loss of Gis1 on *GRE1* expression, we created a new reporter construct, fusing the 778 bp upstream region of the *GRE1* start codon, which contains all six PDS elements of the *GRE1* promoter, to the β-galactosidase encoding *lacZ* ORF in plasmid pLS9. Expression of β-galactosidase from this reporter was monitored throughout the diauxic growth phase in wild-type, *rim15Δ* and *gis1Δ* cells. As already seen on the basis of protein levels in post diauxic phase cells, where Gre1 was hardly detectable in the mutant strains,

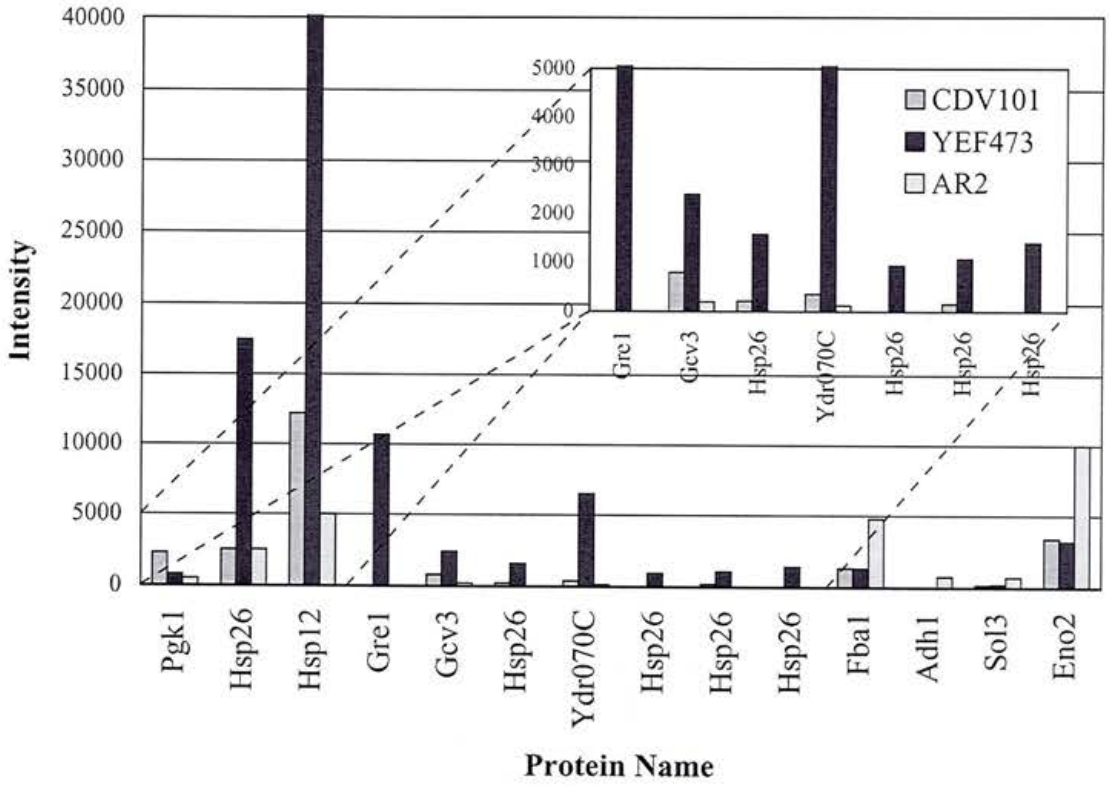

Fig. 7 Expression levels of proteins in stationary phase wild-type (YEF473), *rim15Δ* (AR2), and *gis1Δ* (CDV101) cells as determined by 2D gel-electrophoresis. Spots that differed in expression between the strains were chosen and proteins were identified by MALDI/MS. The insert represents a zoomed part of the chart as indicated by the dashed lines, to better analyze expression patterns of proteins that exhibited low absolute intensities compared to the prominent *HSP12* peak in the main chart.

GRE1-lacZ expression was not induced at any stage of the cellular growth phases (Fig. 8A). In wild-type cells, *GRE1*-lacZ expression is induced just about at the time when glucose becomes exhausted (between time points 19 hrs and 35 hrs, glucose data not shown) and further increases during entry into stationary phase.

Similarly, we constructed a *GRE1-GFP* reporter, where we fused the same promoter region of *GRE1* to *GFP* in the high-copy number plasmid YEplac181. Wild-type cells transformed with this reporter construct incubated for four days on plates started to glow greenish when exitated with an ultraviolet lamp (Fig. 8B). *rim15Δ* and *gis1Δ* cells transformed with the same construct showed only a slight emission, which was also observed in untransformed cells, indicating that the GFP expression was specifically under control of the *GRE1* promoter and no promoter leakage occurred.

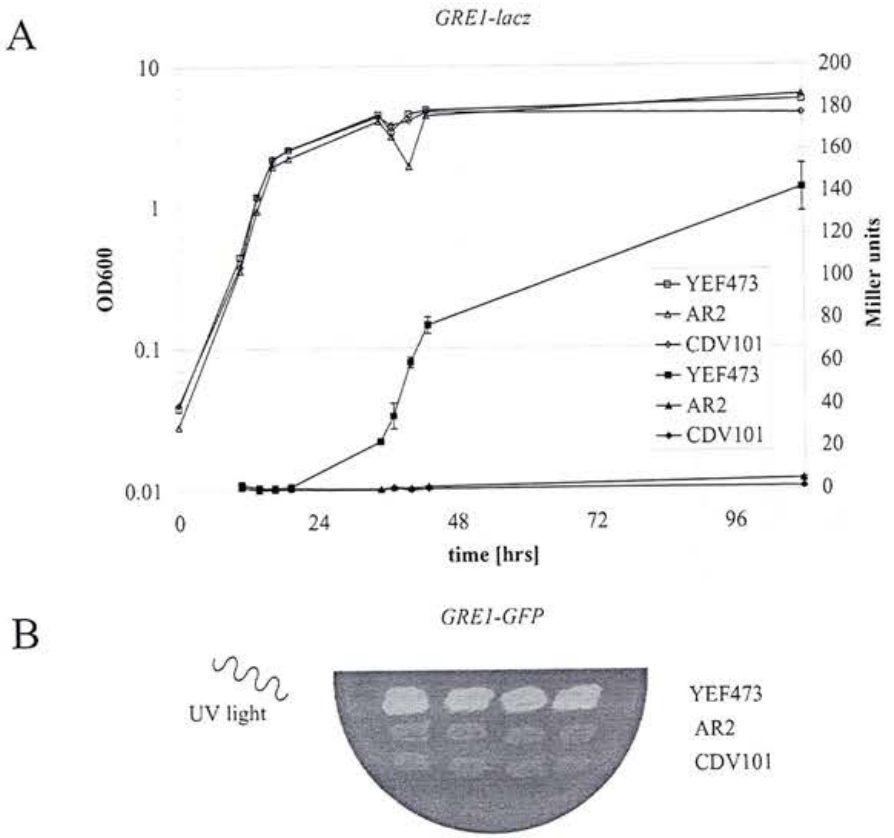

Fig. 8 Expression of a *GRE1-lacZ* reporter in wild-type (YEF473), *rim15Δ* (AR2), and *gis1Δ* (CDV101) cells was assayed throughout the diauxic shift. The strains were grown in YPD (1% glucose) and cell density and β-galactosidase activity was measured at the times indicated **(A)**. The same strains were transformed with a *GRE1-GFP* reporter plasmid and incubated for 4 days on selective SD plates. Expression of the reporter was monitored on a Chemidoc Gel-imaging system (Biorad) using UV as light source **(B)**.

Taken together, the presented analysis of the results from the 2D-gel experiment strongly support our model that Gis1 controls PDS-element dependent transcription and that the transcription factor is under positive control of Rim15 during entry into the diauxic shift phase.

Discussion

We have identified the zinc finger protein Gis1 in a screen for dosage-dependent suppressors of the *rim15Δ* defect in nutrient-limitation induced transcriptional derepression of *SSA3*. Our

results are most simply interpreted in a model in which Gis1 functions in the Ras/cAMP pathway downstream of Rim15 to control transcription of a set of genes whose products are essential for long term survival following nutrient limitation. Several observations support such a model. First, loss of Gis1 causes a defect in transcriptional derepression following nutrient limitation of various genes, including *SSA3,* whose expression is known to be negatively regulated by the Ras/cAMP pathway through Rim15. As a result, *gis1Δ* cells are seriously defective in induction of starvation resistance upon entry into stationary phase. Second, deletion of *GIS1* partially suppresses the growth defect at elevated temperatures of mutant cells that are compromised for PKA activity (*e.g., cdc25^{ts}, cdc35^{ts}*, and *tpk2^{ts}*). Third, overexpression of *GIS1* suppresses the defect in transcriptional derepression of *SSA3* in nutrient-limited *rim15Δ* cells, induces transcription of PKA-repressible genes (*e.g., SSA3, HSP12,* and *HSP26*) in exponentially growing wild-type cells, and exacerbates the growth defect of strains compromised for PKA activity. Fourth, expression driven by the PDS element, which has previously been shown to be negatively regulated by the Ras/cAMP pathway and which confers transcriptional activation following nutrient limitation at the diauxic transition and in stationary phase, is almost entirely dependent on the presence of Gis1.

It was recently reported that most of the growth-related effects of PKA might be accounted for by Msn2/Msn4-controlled transcription (Smith *et al.*, 1998). In this context, our result showing that loss of Gis1 only partially suppresses the growth defect of PKA-compromised mutant cells suggests that Gis1 may rather weakly antagonize PKA-dependent growth. Nevertheless, overexpression of *GIS1*, in particular under conditions of presumably reduced activity of the Ras/cAMP pathway (*e.g.,* on galactose-containing medium), was found to strongly inhibit growth. This indicates that one role of Gis1 is indeed to stimulate the expression of growth inhibitory genes and that PKA-dependent control of growth is the result of a coordinated regulation of various, different transcription factors. Accordingly, PKA might regulate growth through its direct and/or indirect modulation of transcriptional activators including Msn2, Msn4, and Gis1 as well as by its action on transcriptional repressors such as for instance Sok2 (Ward *et al.*, 1995; Smith *et al.*, 1998). Interestingly, although there is as yet no evidence that PKA phosphorylates Msn2 directly, recent results suggest that PKA exerts its effect on Msn2-dependent gene expression by directly inhibiting Msn2 function (Gorner *et al.*, 1998). In this context, our model posits that Gis1 acts downstream of a protein kinase, Rim15, which is negatively controlled by PKA-dependent

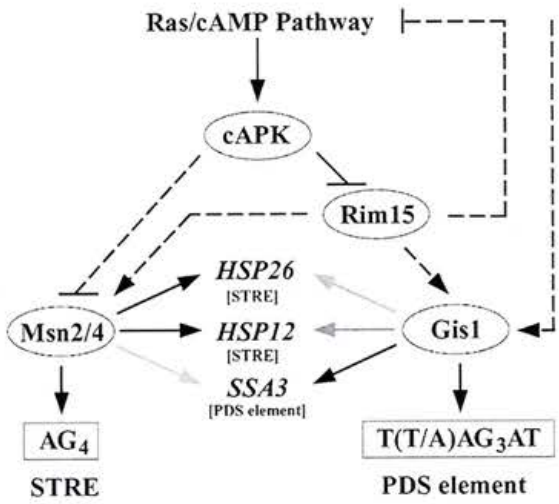

Fig. 9 Model for PKA regulation of nutrient limitation-induced gene expression. The relative importance of Gis1 and Msn2/4 for transcriptional activation of *HSP12, HSP26,* and *SSA3* upon nutrient limitation is illustrated by the different shading (*i.e.,* darker shading representing stronger dependence on the corresponding zinc finger protein; see Martínez-Pastor *et al.*, 1996, for studies of Msn2/4-dependent transcription). Notably, *HSP12* and *HSP26* have several STREs in their promoter regions, while the *SSA3* promoter contains no conserved STRE, but several PDS elements (indicated in brackets). Arrows and bars denote positive and negative interactions, respectively. Dashed arrows and bars refer to potential interactions. For further details, see text.

phosphorylation (Reinders *et al.*, 1998). One might predict, therefore, that Gis1 activity is directly modulated by Rim15-dependent phosphorylation (Figure 9). In accordance with such a model, we found that transcriptional activity of a LexA-Gis1 hybrid protein is partially dependent on the presence of Rim15. Nevertheless, both the one-hybrid assay and the *rim15Δ* suppression experiments indicate that Gis1 may become largely independent of Rim15 when overproduced from the strong constitutive *ADH1*-, or inducible *GAL1*-promoters. This effect may be due to the fact that *GIS1* overexpression yields protein levels that exceed a certain threshold value beyond which Gis1 becomes independent of upstream regulatory proteins. Alternatively, Gis1 may be controlled via additional Ras/cAMP/Rim15-independent mechanisms (Figure 9). Further studies with particular focus on the biochemical characterization of Gis1, which has thus far been hampered by excessive proteolysis of Gis1 (data not shown, see also Jang *et al.*, 1999), should allow a more detailed assessment of this issue.

Loss of Gis1 causes a defect in nutrient limitation-induced derepression/activation of *SSA3, HSP12,* and *HSP26*. Thus, while Gis1 has recently been found to function as a damage-responsive repressor of *PHR1* (Jang *et al.*, 1999), we show here that it formally functions as an activator of *SSA3, HSP12*, and *HSP26*. Such a dual role in transcriptional activation/repression is not uncommon amongst transcriptional regulators and may be based on specific protein-protein interactions, promoter sequence contexts, or physiological conditions (e.g., the repressor-activator Ume6, Malathi *et al.*, 1997; Sweet *et al.*, 1997). Notably, in this context, both our one-hybrid experiments and our overexpression studies suggest that Rph1, unlike its homolog Gis1, may function as a repressor of STRE- and PDS element-controlled genes. Together, these findings support the previous notion that Rph1 and Gis1, in addition to their common role in regulation of *PHR1* expression, may also exert distinct roles in transcriptional control (Balciunas and Ronne, 1999).

Interestingly, *in vitro* footprinting and binding competition studies indicated that Rph1 and Gis1 bind, like Msn2 and Msn4, to STRE-like sequences, suggesting that Gis1 and Msn2/Msn4 may functionally overlap *in vivo* (Treger *et al.*, 1998; Jang *et al.*, 1999). Surprisingly, however, we found that both *msn2 msn4* and *gis1Δ* mutants were specifically defective for STRE-driven and PDS element-driven expression, respectively. Thus, Msn2/Msn4 and Gis1 are not functionally equivalent *in vivo* and are likely to discriminate between the two very similar STRE and PDS promoter elements. Such a conclusion is further supported by our findings that *gis1Δ* mutants are seriously defective for nutrient limitation-induced transcriptional activation of the PDS element-controlled *SSA3* gene, while exhibiting only a minor defect in transcriptional activation of the STRE-controlled *HSP12* and *HSP26* genes under the same conditions. Nevertheless, the observed minor effect of loss of Gis1 on *HSP12* and *HSP26* expression suggests that Gis1 can, possibly in a proper promoter context, also mediate partial STRE-dependent activation. Such context specificity may be masked in heterologous promoter studies because of the absence of potential ancillary sequences that modulate STRE-driven transcription. Therefore, detailed reinvestigation of the *HSP12* and *HSP26* promoters in the context of the present results should allow us to understand the relationship between STREs and their dependence upon the Msn2, Msn4, and Gis1 transcriptional regulators following nutrient limitation.

A further interesting point with respect to the heterologous promoter studies is the finding that *rim15Δ* mutants were partially reduced for nutrient limitation-induced activation of STRE-*lacZ*. One explanation for this result may be that Rim15 is involved in Gis1-independent,

possibly Msn2/Msn4-dependent, control of STRE. Alternatively, Rim15 could, as suggested previously (Reinders *et al.*, 1998), be involved in mechanisms of feed-back inhibition of the Ras/cAMP pathway. In this scenario, loss of Rim15 could cause over-activation of the Ras/cAMP pathway and consequently inhibition of Msn2/4 function. Notably, both explanations are consistent with our model that Rim15 acts upstream of Gis1 and may, therefore, overall cause a more serious physiological defect than loss of Gis1 (Figure 9).

A final issue that remains to be addressed is that Gis1 has previously been implicated in a mechanism of positive regulation of the Ras/cAMP pathway (Balciunas and Ronne, 1999). Accordingly, and at variance with the results presented here, the authors found that overexpression of *GIS1* suppressed a *cdc25-5*^{ts} mutation. It must be noted, however, that the experimental setup in this report differed in at least two important aspects from our studies. First, the *cdc25-5*^{ts} mutant under study, in contrast to the one used here, harbored an additional mutation in the *PDE2* gene whose product, phosphodiesterase, negatively regulates Ras/cAMP signaling (Sass *et al.*, 1986). Second, overexpression of *GIS1* was achieved by expression of *GIS1* under its own promoter from a 2μ plasmid. Presumably, this may yield much lower Gis1 protein levels than expression of *GIS1* from the strong *ADH1*- and *GAL1*-promoters, as done in the present study. One might speculate, therefore, that moderate overproduction of Gis1 in a *cdc25-5*^{ts} *pde2* mutant, while not reaching the threshold level for growth inhibition, causes transcriptional activation of one or more genes that act as specific suppressors of the *cdc25-5*^{ts} mutation. One such candidate gene encodes Tfs1 which, when overproduced, shows allele specific suppression of *cdc25-1*^{ts} and *cdc25-5*^{ts} (Robinson and Tatchell, 1991). Intriguingly, we found the *TFS1* gene to contain a PDS element consensus site at position –136 in its promoter region which may confer the reported induction upon nutrient limitation via the transcriptional activator Gis1 (DeRisi *et al.*, 1997). Despite the admittedly speculative nature of this model, it may reconcile the obviously contradictory observations illustrated above and leads to several predictions that can be tested in future studies.

Identification of a transcription factor that is under positive control of protein kinase Rim15 raises obviously the question of the target genes that are regulated by Gis1. Thanks to a collaboration with Dr. H. Voshol, Novartis, we were able to subject wild-type and *rim15Δ* and *gis1Δ* cells to a comparative 2D gel analysis of protein levels in post-diauxic shift phase cells, when Gis1-dependent transcription is supposed to be active. Spots were identified that showed both a Rim15- and a Gis1-dependent phenotype and the obtained results provided us

important and interesting clues. Amongst the identified gene products we found *HSP12* and *HSP26*, which confirmed our previous results using northern blot analysis (see also Fig. 1). Intriguingly, one of the genes whose derepression was most strongly affected by loss of Rim15 and Gis1, was *GRE1*, which has six PDS element consensus sequences in its promoter region. Analysis of *GRE1-lacZ* and *GRE1-GFP* reporter constructs showed Rim15- and Gis1-dependent transcriptional activation upon nutrient starvation, which is in agreement with our above presented model. The expression of green fluorescent protein instead of β-galactosidase as a reporter gene may offer several advantages for future studies. First, GFP does not need an enzymatic reaction for quantitative analysis and can be rapidly quantitated by fluorometric measurements of liquid cultures. Secondly, screening for β-galactosidase activity on plates supplemented with X-gal relies on leakage of β-galactosidase out of the cell. Accordingly, genes whose products either enhance secretion, or disturb the cell's integrity, will therefore enlarge the part of fake positive clones. Use of a GFP reporter circumvents this problem. With the acquired knowledge in this study and the new reporter constructs, the *GRE1-lacZ* and *GRE1-GFP,* we have further tools to identify new interactors of the Ras/cAMP pathway, that are specifically involved in the processes necessary for entry into stationary phase.

Chapter IV

Identification of a new protein phosphatase type 1 regulatory protein that may positively control Rim15-dependent activation of the stationary phase program

Identification of a new protein phosphatase type 1 regulatory protein that may positively control Rim15-dependent activation of the stationary phase program

Introduction

Analyses of the components of the Ras/cAMP/PKA pathway and their physiological implications have shown that the major role of this pathway is to regulate growth in response to glucose availability, which is signaled via a newly identified GPCR. The pathway serves to sense the presence of glucose and to trigger rapid changes in metabolism that are necessary to utilize glucose, a function which is particularly needed when starved cells reenter fermentable growth after a prolonged resting state. All evidence obtained so far has found that the entire signaling cascade, although it may be modulated at different levels and integrate also other inputs than nutritional signals (e.g. stress signals), converges on one single protein, namely PKA. Active PKA presumably reflects the presence of abundant fermentable carbon source and absence of stress, and promotes proliferative growth and precludes access to stationary phase. Nutrient limitation, on the other hand, causes inactivation of the pathway, which leads to activation of a stationary phase program following G_1 cell cycle arrest, that includes accumulation of storage carbohydrates and induction of stationary phase-specific genes. The pathway is not only inactivated by glucose starvation, but also following several other stress conditions, including heat, osmotic, and oxidative stresses. The different input signals also lead to different cellular adaptations, that can be very specific for a particular condition (*e.g.* PDS element-dependent transcription is induced upon nutrient starvation, but not following heat stress (Boorstein and Craig, 1990, own studies)). It is therefore very likely that there exist additional regulatory mechanisms in parallel to PKA to act on downstream effectors of PKA in response to a specific stimulus.

The protein kinase Rim15, has been shown to be directly and negatively controlled by PKA (Reinders *et al.*, 1998). Rim15 controls a subset of PKA induced phenotypes during the post-diauxic phase and upon entry into stationary phase. Rim15 is inactivated by phosphorylation via PKA during exponential growth and it is possible that activation of Rim15 does not only occur by release from this inhibition, but also by alternative mechanisms that may directly control Rim15 function. The aim of the work presented in this chapter was to identify additional regulatory mechanisms that may control Rim15 activation.

Results

Identification of *BUD14* as an activator of the stationary phase program

To isolate specific activators of the protein kinase Rim15, we screened for genes that, when overexpressed, were able to induce Rim15-dependent activation of a *SSA3-lacZ* reporter in exponentially growing cells. To this end, we used a similar screen setup as described in Chapter II. Wild-type cells (AR1-1A) carrying the *SSA3-lacZ* reporter plasmid pWB204Δ-236 form blue colonies after 3 days on synthetic defined growth medium containing 0.35% glucose and the β-galactosidase substrate X-gal (see also Chapter II, Fig. 2). The wild-type strain was transformed with a yeast genomic library on the high-copy number plasmid pSEY18 (Kunz *et al.*, 1993) and we have isolated transformants that were able to induce formation of blue colonies already after one day on plates. The genomic inserts of isolated plasmids were partially sequenced from both sides and clones bearing genomic inserts with multiple open reading frames (ORF) were further analyzed by subcloning restriction fragments containing the individual ORFs, using endogenous restriction sites, into high-copy number plasmid YEplac181. Subsequently, we determined whether the observed transcriptional activation was dependent on Rim15, by introducing the plasmids into an isogenic *rim15Δ* strain (AR1-1C) and assaying induction of the *SSA3-lacZ* reporter as described. One of the positive clones that showed Rim15-dependent *SSA3* transcription already in an early stage on plates contained the open reading frame *YAR014C/BUD14*. The deduced amino acid sequence of *BUD14*, a protein of unknown function at that time, contains two highly interesting domains. The first domain is similar to the mammalian *Src* homology 3 (SH3) domain, that has been shown to mediate protein-protein interaction (Cicchetti *et al.*, 1992). The second domain consists of a (R/K)X(V/I)XF motif, which is known to serve as a type 1 protein phosphatase (PP1) recognition site (Egloff *et al.*, 1997, Fig.1). The only PP1 found in yeast is encoded by the gene *GLC7*. Glc7 is an essential serine/threonine phosphatase shown to be implicated in the regulation of a diverse array of physiological functions, like glycogen biosynthesis, cell cycle control, sporulation and glucose repression in *S. cerevisiae*. Glc7 exhibits little substrate specificity *in vitro*. It has been proposed that specificity is in part dictated by regulatory subunits that target the catalytic subunit to its site of activation and/or regulate substrate specificity (Cohen and Cohen, 1989). Notably, Glc7 has previously been suggested to interact with the Ras/cAMP pathway (Matsuura and Anraku, 1994), therefore we

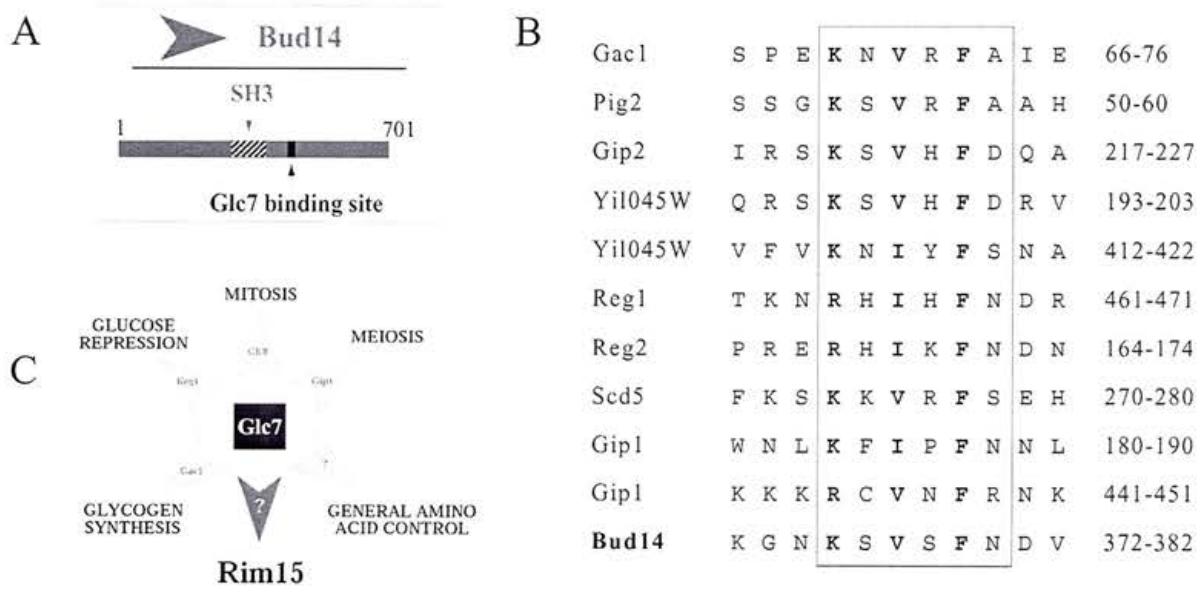

Fig. 1 Bud14 harbors a SH3 domain, known to mediate protein-protein interaction, and a type 1 protein phosphatase (PP1) Glc7 binding site in its deduced amino acid sequence (A). This binding site is represented by a conserved (K/R)X(V/I)XF motif, commonly found in regulatory subunits of Glc7 (B). PP1 is involved in various physiological pathways, and these processes appear to be regulated by distinct PP1 holoenzymes in which the same catalytic subunit (Glc7) is complexed to different targeting or regulatory subunits, to confer substrate specificity (C).

decided to focus our work on elucidation of a possible role of Bud14 and Glc7 as components in nutrient-regulated activation of the stationary phase program.

Bud14 interacts with the type 1 protein phosphatase Glc7

To assess whether Bud14 could function as a regulatory subunit of Glc7, we first investigated the interaction between Bud14 and Glc7. Both two-hybrid and co-immunoprecipitation data confirm that Bud14 specifically interacts with Glc7 (Table I and Fig. 2). Additionally, since complete loss of Glc7 cannot be studied, because *GLC7* is an essential gene, we included in our studies a set of known *glc7* mutants that were produced in an alanine scanning mutagenesis (Baker *et al.*, 1997). Interestingly, we found that the Glc7-133 protein had a defect in its association with Bud14, as confirmed by both two-hybrid and co-immunoprecipitation experiments. Moreover, we constructed two mutant proteins of Bud14 where we replaced two of the conserved amino acids of the predicted Glc7 binding motif by alanine (i.e. Bud14^{V377A} and Bud14^{F379A}; see also Fig. 1). These mutant proteins were also

Table I. Two hybrid interaction between Bud14 and Glc7

	pJG4-5 (AD)				
pEG202 (DBD)	**Bud14**	**Bud14^{V377A}**	**Bud14^{F379A}**	**Rim15**	**Msb2**
Glc7	**2098.4**	5.18	45	2.82	12.7
Glc7-127	**1816.9**	n.d.	n.d.	3.06	2.1
Glc7-132	2.57	n.d.	n.d.	1.93	1.7
Glc7-133	18.7	n.d.	n.d.	1.43	2.3
Bud14	6.26	n.d.	n.d.	2.72	184.8
Msb2	23.3	5.09	5.17	15.6	14.7

Interactions between the indicated proteins were detected using the two-hybrid system. Numbers represent mean β-galactosidase activities (in Miller units) from at least three independent transformants for each pair of plasmids. The strong interaction found between the wild-type Bud14 and Glc7 proteins (and one of its mutant versions) is indicated in bold.

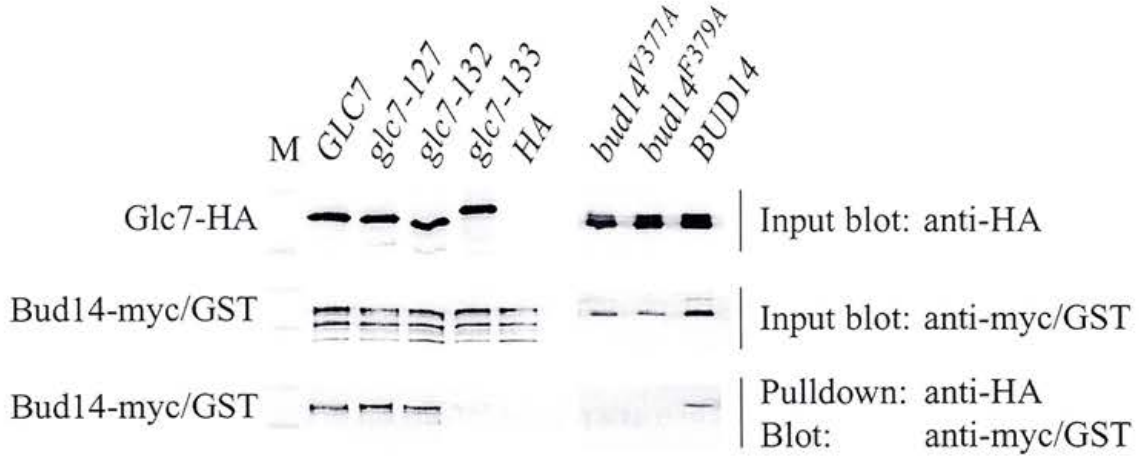

Fig. 2 Co-immunoprecipitation experiments between epitope-tagged Glc7-HA and Bud14-myc (for the different *GLC7* mutant alleles) or GST-Bud14 (for the different *BUD14* mutant alleles) proteins, respectively, were performed by immunoprecipitating proteins from whole cell extracts of IP19 transformed with YCpHA22-*GLC7*, YCpHA22-*glc7-127*, YCpHA22-*glc7-132*, YCpHA22-*glc7-133*, and YCpHA22, respectively, and of KT1961 cotransformed with YCpHA22-*GLC7* and YCpIF2-*GST-bud14^{V377A}*, YCpIF2-*GST-bud14^{F379A}*, and YCpIF2-*GST-BUD14*, respectively, with an anti-HA antibody. Precipitated proteins were analyzed by immunoblotting. The relevant mutant strains used in the experiment are indicated above the individual lanes, the detected, tagged proteins are indicated on the left.

found to be defective for interaction with Glc7. These results support the idea, that Bud14 indeed specifically binds to the catalytic subunit Glc7, and that the predicted Glc7 binding site is required for this interaction.

BUD14 is required for proper entry into stationary phase

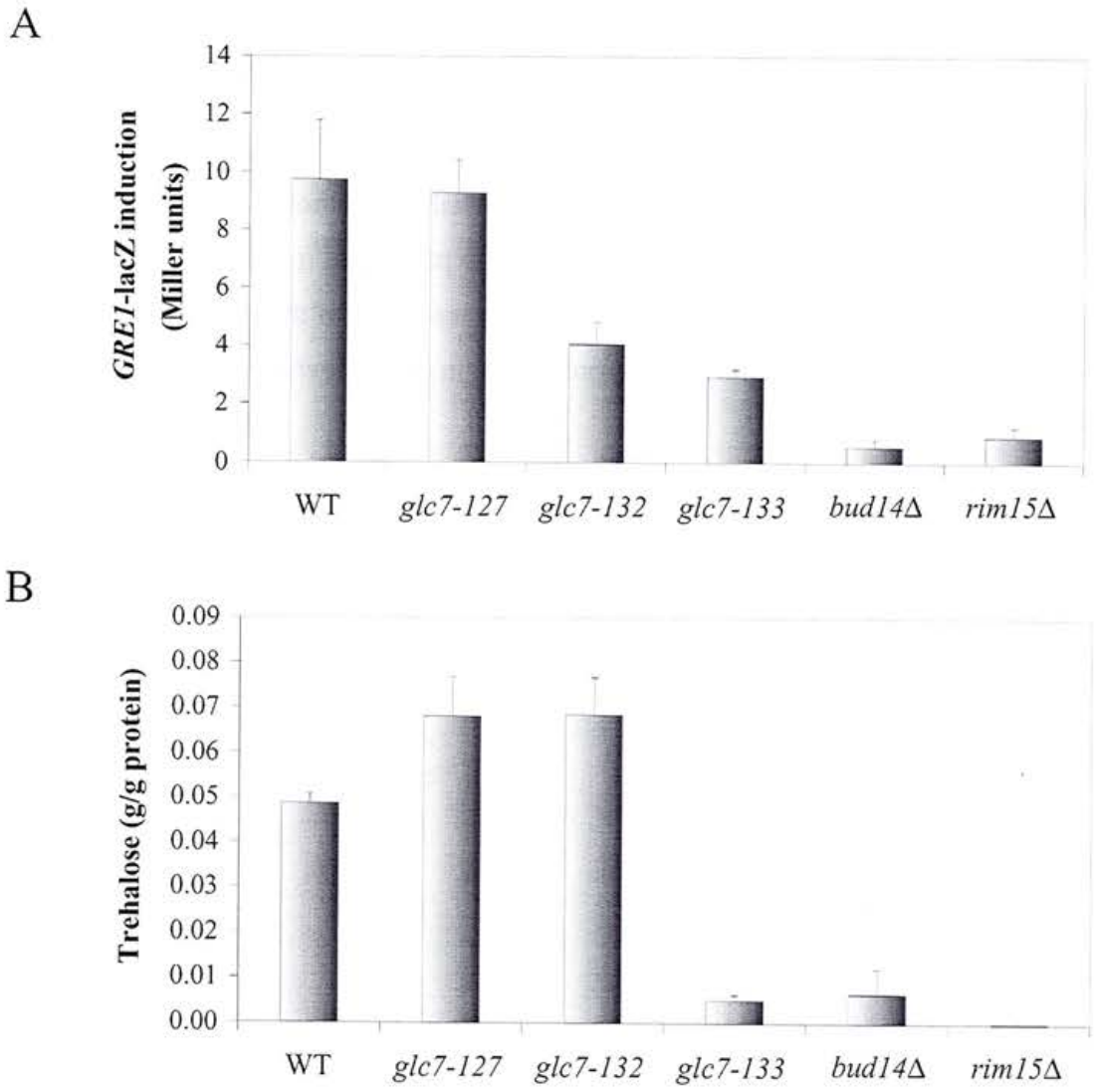

Fig. 3 Effects of *glc7*, *bud14*, and *rim15* mutations on expression of a *GRE1-lacZ* reporter gene **(A)** and trehalose accumulation **(B)** in stationary phase. Wild-type (KT1960), *glc7-127* (KT1708), *glc7-132* (KT1703), *glc7-133* (KT1706), *bud14Δ* (CDV158), and *rim15Δ* (IP31) cells were grown for 87 hrs on YPD (1% glucose) medium. β-galactosidase activities were measured to monitor the induction of the *GRE1-lacZ* fusion gene, and trehalose concentrations were determined as described.

To study the function of Bud14 and its potential interaction with the Ras/cAMP pathway, we constructed a *bud14Δ* mutant. Analysis of the *bud14Δ* mutant revealed that it fails, like the *gis1Δ* and *rim15Δ* mutants, to induce transcription of a *GRE1-lacZ* reporter upon entry into post-diauxic phase (Fig. 3A; notably, the *GRE1* promoter contains 6 PDS elements, which have previously been shown to mediate Rim15- and Gis1-dependent transcription). Similar effects were observed for trehalose accumulation upon entry into the post-diauxic phase (Fig. 3B). Interestingly, the *glc7-133* mutant, which has a defective Glc7-133-Bud14 interaction, shows a similar phenotype as both *bud14Δ* and *rim15Δ* cells. This supports a model in which Bud14 may be a regulatory subunit conferring specificity of the PP1 Glc7 towards a compound of the Ras/cAMP pathway.

In parallel to its defects in *GRE1-lacZ* expression and trehalose synthesis, the *bud14Δ* mutant, similar to the *rim15Δ* mutant, was also highly sensitive to prolonged nutrient starvation (Fig. 4). These phenotypes are commonly associated with hyperactivity of the Ras/cAMP pathway, and suggests that Bud14 seems to play an important role in either downregulating the activity

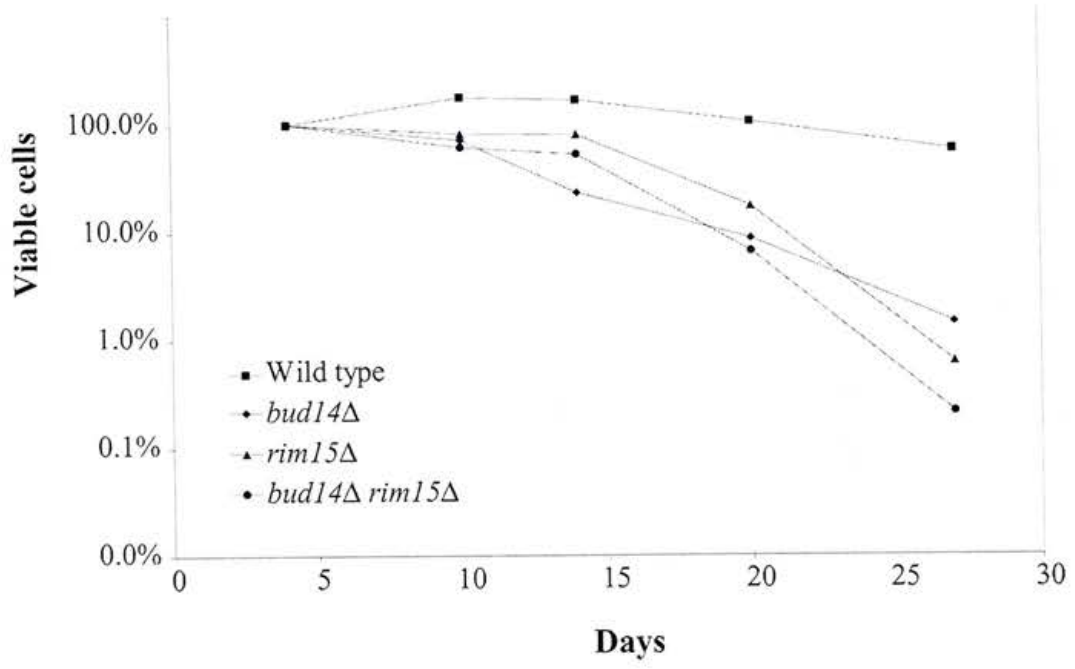

Fig. 4 Effects of loss of Bud14 and loss of Rim15 on stationary phase survival. Wild-type (KT1961), *bud14Δ* (CDV158), *rim15Δ* (IP31)and *bud14Δ rim15Δ* (IP36-1A) mutant cells were grown to stationary phase on YPD medium. The percentage of viable cells was determined by the colony-forming efficiency on YPD agar at the times indicated.

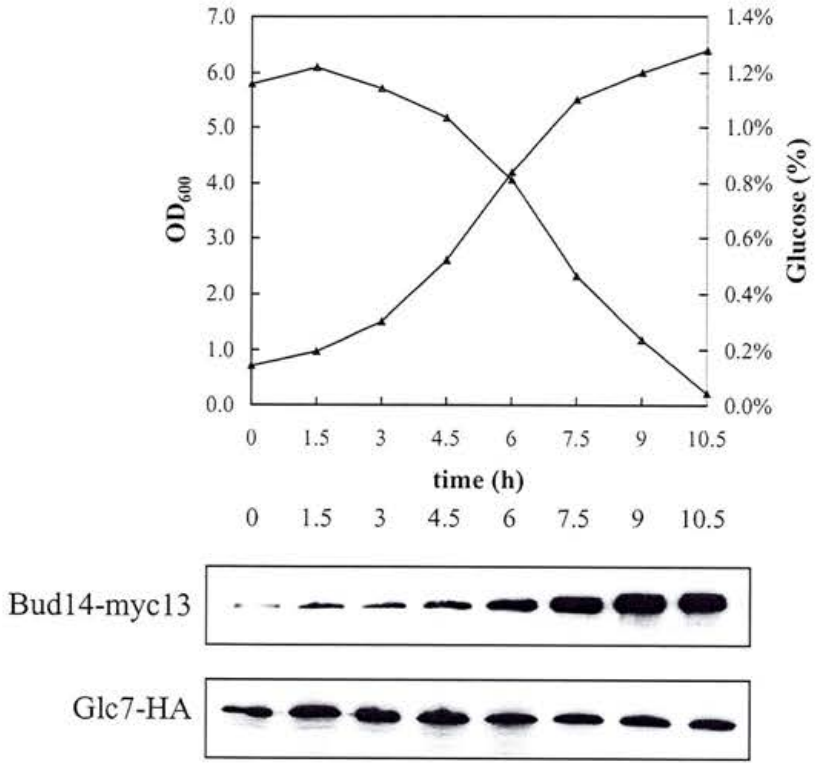

Fig. 5 Protein levels of Bud14 and Glc7 during the diauxic growth phase. Strain CDV138 was grown in YPD and samples were taken at the times indicated. Remaining glucose concentration in the culture medium was determined and protein levels were assessed by immunoblotting whole cell extracts with anti-myc and anti-HA. Cell extracts were normalized by measuring protein concentration using Bradford and verified by Coomasie brilliant blue staining (not shown).

of the pathway or in induction of effectors upon entry into stationary phase. Interestingly, in this context, protein levels of Bud14-myc increase during the diauxic-phase of growth. While the protein levels during exponential growth are relatively low, the amount of Bud14 protein starts to increase just when about half of the glucose is depleted (Fig. 5), and reaches a steady level in resting cells. This again, supports a role for Bud14 in induction and maintenance of the stationary phase program. Glc7-HA protein levels were found to be constant throughout the experiment, as expected for an abundant catalytic subunit that is also involved in various cellular nutrient-independent processes.

Loss of Bud14 partially suppresses *tpk*^{ts}

To further elucidate whether Bud14 may interact with compounds of the Ras/cAMP pathway, we determined if the loss of Bud14 could suppress the phenotype of reduced levels of PKA in a *tpk1Δ tpk2-63 tpk3Δ* temperature sensitive strain (*tpk*^{ts}). Low levels of PKA activity in a *tpk*^{ts} strain at semi-permissive temperatures causes cells to grow at a reduced rate, most likely due to induction of growth inhibitory effectors normally repressed by PKA during exponential growth. Notably, deletion of *BUD14* restored the growth rate defect at 31°C of a *tpk*^{ts} mutant to wild-type levels (Fig. 6A) and partially suppressed the induction of a *GRE1-lacZ* reporter gene that is observed in the *tpk*^{ts} mutant following exposure of the cells to 34°C (Fig. 6B). Thus, loss of Bud14 partially releases cells from the effects of reduced PKA activity, and antagonizes PKA-dependent proliferation control.

Overexpression of *BUD14* exacerbates the growth defects of *cdc25*^{ts}, *cdc35*^{ts} and *tpk*^{ts} strains

One possible interpretation of the results presented above is that Bud14 may act as an activator of Rim15. In accordance with such a model, we found that Bud14 overproduction

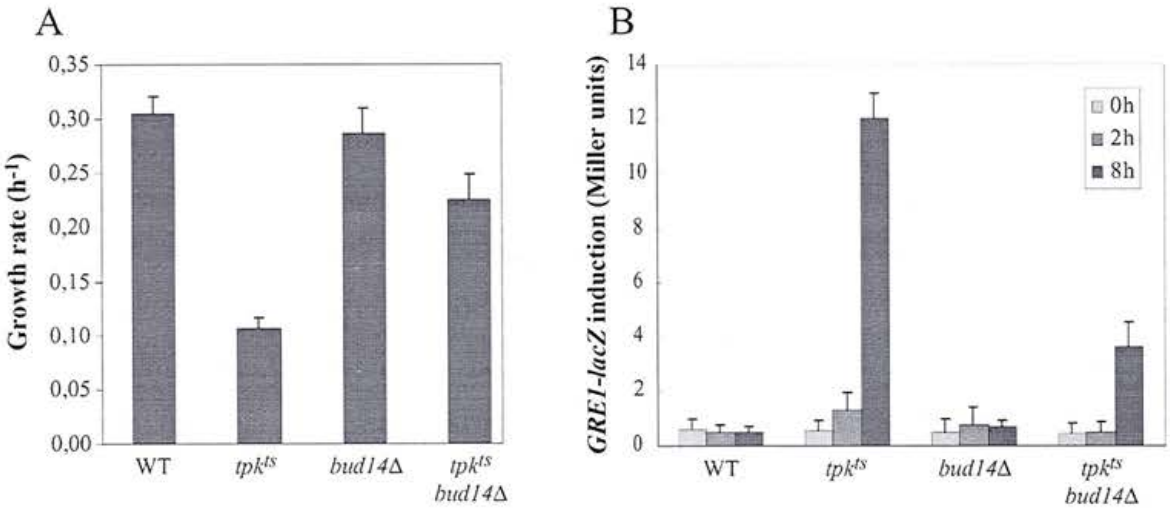

Fig. 6 Growth rates of wild-type (ASY18), *tpk*^{ts} (SGY446), *bud14Δ* (PE12), and *tpk*^{ts} *bud14Δ* (PE15) strains at 31°C on YPD medium were calculated by measuring the optical density of three independent liquid cultures per strain at given time intervals. Values are displayed as generations per hour (A). Induction of a *GRE1-lacZ* reporter gene was measured after shifting cells to 34°C for 0, 2, and 8 hrs as indicated (B).

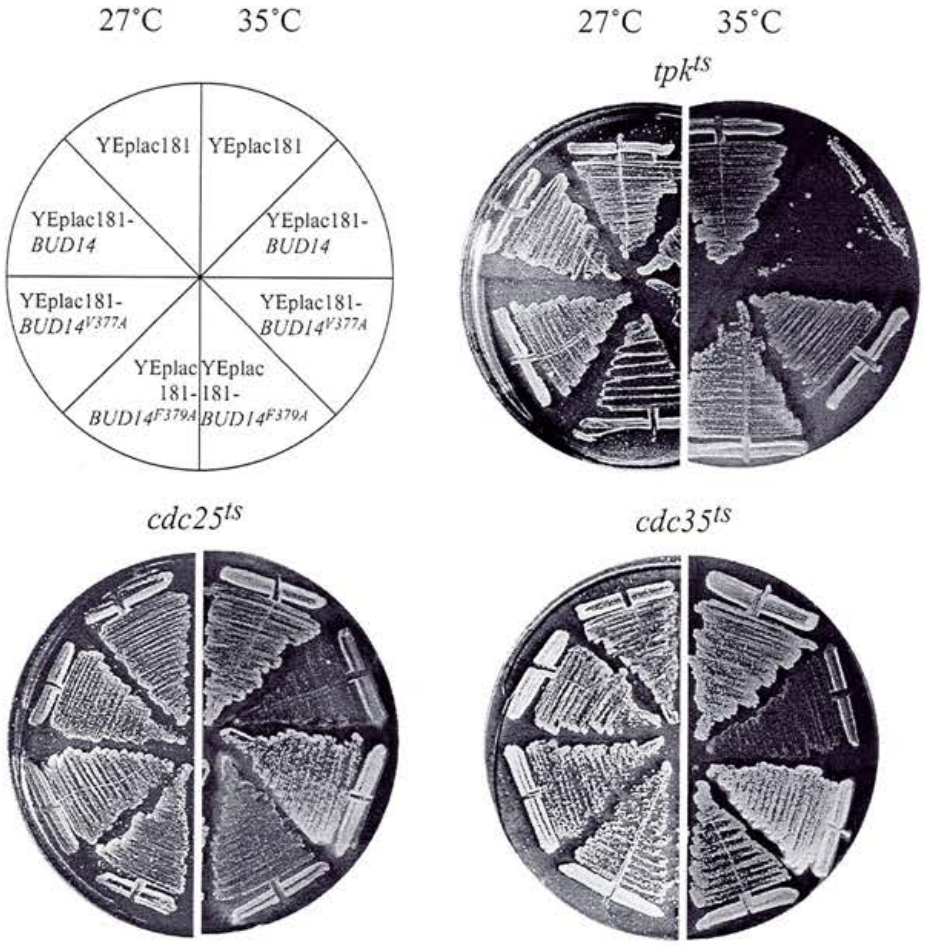

Fig. 7 The effect of Bud14 overproduction on cell growth of *cdc25*^{ts}(OL86), *cdc35*^{ts}(PD6517), and *tpk*^{ts}(SGY446) strains was assessed by streaking the strains on YPD plates and incubating for 3 days at the permissive temperature of 27°C and the semi-permissive temperature of 35°C, as depicted in the schematic.

exacerbated the growth defect of strains with reduced PKA activity; i.e. overproduction of Bud14 (like overproduction of Rim15) caused a severe growth defect in *cdc25*^{ts}, *cdc35*^{ts} and *tpk*^{ts} strains already at a semi-permissive temperature of 35°C (Fig. 7). Notably, overproduction of Bud14^{V377A} and Bud14^{F379A} mutant proteins, which are specifically defective for their interaction with Glc7, did not exacerbate the growth defect of the corresponding *cdc25*^{ts}, *cdc35*^{ts} and *tpk*^{ts} strains.

PP1 influences the phosphorylation state of Rim15

The analysis presented above of the epistatic relationship between Bud14 and the Ras/cAMP pathway suggests that the Bud14/Glc7 module may antagonize PKA-mediated cell proliferation control. Since the *glc7-133*, *bud14*^{V377A}, and *bud14*^{F379A} mutant alleles, which specifically disrupt the interaction between the corresponding Glc7 and Bud14 proteins, were found to cause similar defects upon entry into stationary phase as *rim15Δ*, a possible function of Bud14/Glc7 may be the specific activation of Rim15. To test such a model, we investigated whether Bud14 or Glc7 may have an effect on Rim15 activity *in vitro*. To this end, we isolated affinity tagged GST-Rim15 from wild-type and *bud14Δ* mutant cells. The entire

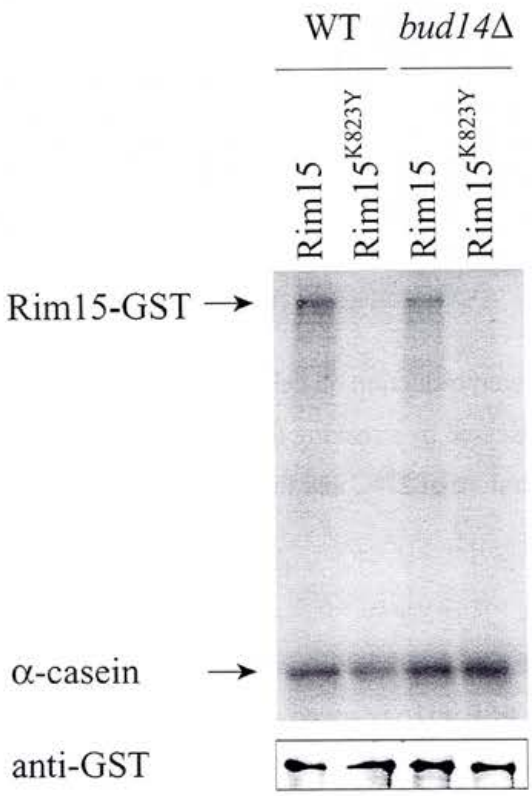

Fig. 8 Influence of Bud14 on kinase activity of Rim15. GST-tagged versions of the protein kinase Rim15 and a 'kinase dead' mutant Rim15^{K823Y} were isolated from wild-type (CDV147) and *bud14Δ* mutant (IP38) cells in the presence of phosphatase inhibitors and analysed for autophosphorylation and α-casein phosphorylation activity. Equal amounts of the fusion protein (verified by immunoblotting with anti-GST antibodies) were incubated with [γ-^{32}P]ATP as described in Materials and Methods and phosphorylation levels were detected by autoradiography.

purification procedure was performed in the presence of phosphatase inhibitors, to prevent unspecific phosphorylation/dephosphorylation of Rim15 during the isolation procedure and consequently trap the protein in the activation state present in the cell at the time of harvest. To assess the amount of Rim15-dependent phosphorylation, we also isolated in parallel an ATP-binding deficient GST-Rim15^{K823Y} mutant protein known to have strongly reduced kinase activity (Reinders *et al.*, 1998). Phosphorylation of Rim15 was completely absent in the Rim15^{K823Y} mutant (Fig. 8). Thus, the major part of Rim15 phosphorylation is due to autocatalytic phosphorylation activity of Rim15. Phosphorylation of the exogenous substrate α-casein was equally strong both in GST-Rim15 and GST-Rim15^{K823Y} preparations, indicating that α-casein phosphorylation is independent of Rim15 kinase activity, and probably caused by a contaminating, co-purified kinase. Therefore, we only compared the autophosphorylation pattern of Rim15 isolated from wild-type or *bud14Δ* cells. Accordingly, Rim15 kinase activity appears only slightly reduced in a *bud14Δ* strain and may not be modulated by Bud14/Glc7 to an extend necessary to explain the effects seen due to a loss of Bud14.

To further elucidate the effect of Bud14/Glc7 on Rim15, we investigated the effect of PP1 activity on Rim15 kinase activity. Co-immunoprecipitation attempts to precipitate Glc7-HA and GST-Rim15 showed, that Glc7-HA coprecipitates with GST-Rim15 glutathione-sepharose beads, but also with beads alone. Thus, in these experiments, GST-Rim15 glutathione sepharose beads also contain Glc7-HA. Thus, we looked at the effect of a specific type 1 protein phosphatase inhibitor (I-1) on Rim15 kinase activity. At low I-1 concentrations, the autophosphorylation band of Rim15 was found to be reduced, but at increasing concentrations of the inhibitor, autophosphorylation of Rim15 increased to levels even higher

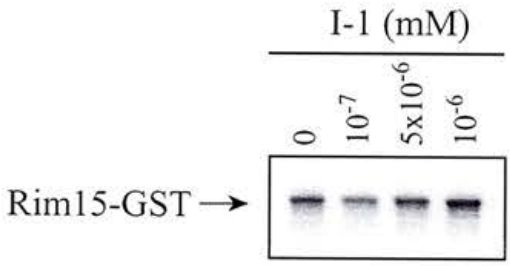

Fig. 9 PP1 activity alters GST-Rim15 phosphorylation pattern. GST-tagged Rim15 was incubated with [γ-32P]ATP in the presence of Glc7 and increasing amounts of PP1 Inhibitor I-1. Phosphorylation levels were deteced by autoradiography.

than in the control without I-1 inhibitor (Fig. 9). One possible explanation for this observation is that the PP1 inhibitor prevents in vitro activation of Rim15 by PP1 at low, physiological conditions. At elevated levels however, complete inactivation of Glc7 inhibits removal of phosphates from Rim15, which, albeit with a lower activity, continues to exert its autocatalytic phosphorylation, resulting in an increase of radiolabelled phosphates on Rim15 over time. Alternatively, Glc7-dependent dephosphorylation may not regulate Rim15 activity, but rather its interaction with potential target proteins or, alternatively, its subcellular localization.

Taken together, inhibition of Glc7 has a measurable effect on the phosphorylation state of Rim15 *in vitro*. The consequences of Glc7-dependent dephosphorylation for the function of Rim15 *in vivo* will still have to be elucidated in more detail.

Discussion

The mechanisms how PKA activity is regulated in response to the different environmental stimuli has been elucidated during the last decade in increasing detail. The picture that converged from various studies is that PKA-dependent readouts may themselves be subject to additional control mechanisms that act in parallel to the Ras/cAMP pathway and that they converge on a given PKA target. For instance, it is most likely that activation of the stationary phase program is not only due to release of growth inhibitory proteins from negative regulation by PKA, but also implicates activatory mechanisms that that control the corresponding proteins positively and impinge on specific PKA targets. These mechanisms may act in parallel to the PKA pathway. In this context, more and more signal transduction pathways known to respond to other signals than glucose, but leading to similar or even overlapping effects have been reported to interact with, or act in conjunction with the Ras/cAMP pathway. One example is the recently described FGM pathway (Crauwels *et al.*, 1997), which seems to modulate PKA activity independently of the classical, cAMP-driven activation. Until now, however, a direct interaction of a parallel pathway with components of the Ras/cAMP pathway or any of its effectors has not unequivocally been shown.

Here, we report the identification of a novel PP1 regulatory subunit Bud14, that interacts with the Ras/cAMP pathway to mediate entry into stationary phase following inactivation of the pathway. This is possibly exerted by dephosphorylation of the protein kinase Rim15, an activator of the stationary phase program that is under negative control of PKA. Bud14 was

isolated as a multicopy activator of a *SSA3-lacZ* reporter in an early stage of cell growth, a phase were *SSA3* is normally repressed by PKA. This activation was dependent on the presence of Rim15, suggesting that Bud14 acts upstream of Rim15 in the Ras/cAMP pathway, or in a parallel pathway that converges on Rim15. In accordance with such a model, *bud14Δ* mutants fail, like *rim15Δ* mutants, to induce expression of a *GRE1-lacZ* reporter gene. *GRE1* has been shown to be specifically activated by Rim15 via PDS element-dependent transcription through the transcription factor Gis1. Additionally, *bud14Δ* mutants fail to accumulate trehalose and, possibly as a consequence, are strongly defective in induction of starvation resistance upon entry into stationary phase. Overexpression of Bud14, like Rim15, exacerbates the growth defect of PKA compromised strains such as the temperature sensitive mutants of Cdc25, adenylate cyclase, or PKA itself. This, again, indicates that Bud14 may interact with the Ras/cAMP pathway at the level of or downstream of PKA. In this context, we also found that loss of Bud14 restored the growth defect of strains with reduced levels of PKA and partially suppressed the induction of a *GRE1-lacZ* reporter in the same strain. Taken into considerations, that Rim15 is present in these cells, but inactivation of PKA does not confer Rim15-dependent phenotypes, one could argue that Bud14 acts downstream of Rim15 to control growth inhibitory genes, but this is at variance with the above mentioned necessity of Rim15 for Bud14-mediated activation of *SSA3* transcription in logarithmically growing cells. A model taking into account both observations is that Bud14 acts as an activator of Rim15. Release of Rim15 from negative regulation upon inactivation of the Ras/cAMP pathway would therefore not be enough to fully activate Rim15 function. An additional, possibly Ras/cAMP-independent, on nutrient limitation induced mechanism, may be necessary for activation of Rim15. Loss of a formal activator of Rim15, such as Bud14, may suppress the otherwise growth inhibitory effects of a release from PKA-inhibition.

Little is known about the biochemical properties or enzymatic activities of Bud14. During our studies, it has been proposed to play a role in bud site selection (Ni and Snyder, 2001). Nevertheless, our own results assign to it an important role in induction of stationary phase upon nutrient limitation. Accordingly, Bud14 levels in batch culture show a growth phase related pattern, being highest after the diauxic shift and being maintained high during the post-diauxic phase and in resting cells. On the other hand, the two proposed models do not exclude each other, and it may well be that Bud14 exerts a different role in logarithmically growing cells, and, upon nutrient starvation, fulfills a task with respect to nutrient signaling. Further elucidation and dissection of the putative dual role of Bud14 will have to include the

regulatory mechanism by which the activity of Bud14 may be modulated, something that has not been addressed yet.

The predicted amino acid sequence of Bud14 contains two interesting domains, which provide some clues with respect to its biochemical function. First, it contains a SH3 domain, known to mediate protein-protein interactions via proline-rich sequences in the target proteins (Cicchetti *et al.*, 1992). Notably, Rim15 contains several proline-rich stretches in its amino acid sequence. Secondly, it contains a conserved motif for a PP1 recognition site commonly found in PP1-binding regulatory subunits, a motif found to be invariant from yeast to humans (Egloff *et al.*, 1997). PP1 is one of the major serine/threonine-specific protein phosphatases of eukaryotic cells. In yeast, *GLC7* encodes for the only PP1 present in this organism and it is indispensable for cell proliferation. This enzyme is involved in various cellular processes like glycogen synthesis, glucose repression, mitosis, meiosis and general amino acid control (Stark, 1996). These different processes appear to be regulated by distinct PP1 holoenzymes in which the same catalytic subunit, encoded by *GLC7*, is complexed to different targeting or regulatory subunits. These subunits act to confer *in vivo* substrate specificity not only by directing PP1 to the subcellular compartments of its substrate, but also by enhancing or suppressing its activity towards the different substrates. In addition, the regulatory subunits allow the activity of PP1 to be modulated by reversible protein phosphorylations and second messengers in response to extracellular stimuli. In this context, Bud14 may present a novel PP1 regulatory subunit that targets PP1 to Rim15. In support of this model, Bud14 associates with Glc7 both in two-hybrid and in co-immunoprecipitation experiments. Unfortunately, we were up to now not able to show that Bud14 and/or Glc7 directly interact with each other *in vitro*.

Mutant alleles of *GLC7* have proven valuable in identifying PP1 regulatory proteins and physiological pathways that require Glc7. For example, *glc7-1* and $glc7^{T152K}$ have specific defects in glycogen synthesis (Feng *et al.*, 1991; Francois *et al.*, 1992; Cannon *et al.*, 1994) and glucose repression (Tu and Carlson, 1995), respectively. The product of *glc7-1* is defective in its association with Gac1 (Stuart *et al.*, 1994) while the product of $glc7^{T152K}$ is defective in its interaction with Reg1 (Tu and Carlson, 1995). Accordingly, the phenotypes of *glc7* alleles that specifically disrupt the unity of Glc7 to a particular regulatory protein, are similar to the phenotypes of loss of that particular regulatory protein. In this context, we were also able to identify a *glc7-133* allele, which, similar to the *bud14Δ* mutant, was defective in induction of a *GRE1-lacZ* reporter and in accumulation of trehalose upon nutrient starvation.

Two-hybrid data and co-immunoprecipitation data revealed that this allele is indeed impaired for its association with Glc7. The correlation of this allele-specific defect of *glc7-133* and the null mutant phenotype of the *bud14Δ* mutant provides strong evidence that Bud14 may be the regulatory subunit that regulates phosphatase specificity *in vivo*. In a similar experiment, specific mutations in the PP1 recognition site of Bud14 rendered the protein unable to interact with the catalytic subunit Glc7. Overproduction of these mutant proteins, unlike wild-type proteins, failed to exacerbate the growth defects in strains with reduced PKA activity, suggesting that Bud14 may regulate Glc7 function.

Our genetic data provide quite convincing evidence that the Bud14/Glc7 holoenzyme interacts with the Ras/cAMP pathway and that PP1 may be involved in dephosphorylation mediated activation of Rim15. In a most simple model, Glc7 would act on the same phosphorylation sites than PKA to antagonize inhibition by PKA phosphorylation. Activity of Rim15 would, therefore, be modulated by a balance between phosphorylation and dephosphorylation through PKA and PP1, respectively, and the phosphorylation state of Rim15 determines its kinase activity. Kinase activity assays of Rim15 to verify such a model proved to be difficult in their setup, because isolations often contained a contaminating kinase activity that caused Rim15-independent phosphorylation of the exogenous substrate α-casein. In the absence of a known kinase target, autophosphorylation activity of Rim15 may serve as a means to quantitate Rim15 activity. This includes, however, the caveat that autophosphorylation may directly have a positive or negative effect on Rim15 kinase activity itself.

Rim15 kinase activity is not significantly impaired in a *bud14Δ* mutant strain, where we would expect that loss of the regulatory subunit Bud14 prevents Rim15 from being activated by PP1, ultimately leading to the observed phenotypes that are similar to the complete loss of Rim15. This result, however, may be due to experimental limitations. First, the applied extraction procedure may not have trapped Rim15 in its activation state and Rim15 may have been modulated after extraction *in vitro*. In this context, possibly co-precipitating kinases or other components in our assay, may subject Rim15 during the extraction procedure. Second, the phosphatase inhibitor may interfere with the kinase reaction, because autophosphorylation levels were significantly lower in the presence than in the absence of the inhibitor mix. To avoid this problem, we chose to use a mammalian PP1 inhibitor peptide, I-1, that has been shown to specifically inhibit Glc7 activity when expressed in yeast by competing with the binding of the regulatory subunits (Zheng *et al.*, 2000). Addition of this I-1 inhibitor peptide did not prevent *in vitro* activation of Rim15 to an extend that could explain the drastic

phenotypes of *bud14Δ* or *rim15Δ* mutants. The kinase assays revealed, however, another effect that leads to a somewhat diverging, but not less interesting model. At increasing concentrations of the inhibitor, the phosphorylation caused by Rim15 autocatalytic activity even increased. This suggests that Glc7 exhibits dephosphorylation activity on Rim15, and that complete inactivation of Glc7 leads to increased phosphorylation levels of Rim15 due to the absence of dephosphorylation activity that removes autocatalytically added P_i. This model suggests the presence of a regulatory mechanism for Rim15 other than modulation of its kinase activity, that could involve regulation of substrate specificity or subcellular localization mediated through Bud14/Glc7-dependent dephosphorylation. In such a model, Bud14/Glc7 would act on different phosphorylation sites than PKA, that has been clearly shown to inhibit kinase activity. This is also supported by a previous finding, that a Rim15 mutant allele, where all putative PKA phosphorylation sites have been mutated to alanine, became unresponsive to PKA inhibition, but did not produce the expected constitutively active phenotype (De Virgilio, personal communication). Absence of the required stimulatory mechanism under non-inducing conditions, *e.g.* in logarithmically growing cells, prevents this $Rim15^{5(RRXS/A)}$ mutant from acting on its substrates. Taken together, our results suggest that the phosphorylation sites on Rim15 targeted by PP1 are the same as those for Rim15 autophosphorylation. Rim15 could therefore be subjected to constant negative feedback inhibition by its own autocatalytic activity. Hence, elevated levels of Bud14 after the diauxic-transition, as observed here, would be necessary for maintenance of the active Rim15 state.

Chapter V

Conclusions and perspectives

Conclusions and perspectives

The Ras/cAMP pathway in *S. cerevisiae* links two vital task of the cell together. The first one is perception of the environment, be it the quality and quantity of available nutrients - especially carbon sources – under good growth conditions, or the rapid sensing of adverse conditions like heat, osmotic stress and drought. The second one is the control of the cell cycle and its coordination with growth. As mentioned before, the cell has to coordinate a multitude of biochemical functions needed for size control, regulation of DNA content and distribution, bud formation, separation and cytokinesis. To this end, a cell is reliant on the proper transmission of the extracellular, environmental conditions to fine tune these internal processes. A malfunctioning of this signal transduction process often has deleterious effects for the cell. The Ras/cAMP pathway serves as the signal transduction pathway to coordinate cellular adaptations in response to nutritional conditions. The components of this pathway have been well established (see also Chapter I) and in the last years, many efforts have been made to elucidate the two remaining critical questions, namely how the environmental signal is sensed by the cell and how activation/inactivation of the pathway acts on the cell cycle machinery. Recent findings led to the discovery of a GPCR/G protein receptor system involved in glucose sensing. It presents a further step in elucidation of the sensing mechanisms of the Ras/cAMP pathway and brought the yeast system back in line with the mammalian system, where adenylate cyclase is activated by heterotrimeric G proteins. Motivated by these interesting findings, we focused our efforts to the second question, how the Ras/cAMP pathway regulates cell proliferation. The aim of the present Ph.D. thesis was to identify new components of the Ras/cAMP pathway downstream of PKA that are involved in regulation of these processes. Downstream targets of PKA that are already known include the transcription factors Msn2 and Msn4, that control STRE-dependent transcription of genes under various stress conditions. Additionally, A. Reinders, a former Ph.D. student in our lab, had isolated a protein kinase Rim15 as an interactor of Tps1 when trying to identify regulators of Tre6P synthase. Loss of Rim15 caused not only a defect in accumulation of trehalose in stationary phase, but also a highly pleiotropic phenotype under various stress conditions, some of which overlap with Msn2/4-dependent phenotypes. In her thesis, A. Reinders could show that Rim15 is a direct downstream target of PKA which controls several aspects needed for entry into stationary phase (Reinders *et al.*, 1998).

The correlation mentioned above between some of the Msn2/4-dependent and Rim15-dependent phenotypes, *e.g.* the positive regulation of several stress-induced genes like *HSP12* and *HSP26* suggested that Rim15 may directly act on these transcription factors. We could demonstrate that STRE-dependent expression mediated by Msn2 and Msn4 is independent on Rim15, suggesting the presence of additional Rim15 targets. For this reason, we decided to screen for additional activators and/or effectors of Rim15. The obtained results revealed important new insights into the signaling events downstream of PKA as well as a new activator branch that may feed into the Ras/cAMP pathway.

In a first part, a screen for dosage-dependent suppressors of a *rim15Δ* mutant defect in derepression of *SSA3* upon nutrient limitation provided us with a set of potential effectors of Rim15 (see Chapter II). One of these clones contained a gene that encodes the zinc finger protein Gis1. Interestingly, we found that loss of Gis1 resulted in a defect in transcriptional derepression upon nutrient limitation of various genes that are negatively regulated by the Ras/cAMP pathway, like for instance *HSP12*, *HSP26* and *SSA3*. Test of epistasis as well as transcriptional analysis of Gis1-dependnet expression indicated that Gis1 may act in this pathway downstream of Rim15 to mediate transcription from the previously described PDS element (Boorstein and Craig, 1990). Accordingly, deletion of Gis1 partially suppressed, and overexpression of Gis1 exacerbated the growth defect of mutant cells compromised for PKA activity. Moreover, PDS element-driven expression, but not STRE-dependent transcription, both negatively regulated by the Ras/cAMP pathway and induced upon nutrient limitation, was found to be almost entirely dependent on the presence of Gis1. These results represent a major contribution to the debate on cAMP-dependent control of gene transcription (Martinez-Pastor *et al.*, 1996; Boy-Marcotte *et al.*, 1998; Parrou *et al.*, 1999). STRE-dependent transcription is induced upon various kinds of stresses, and plays thereby an important role in the general stress response. PDS element-dependent transcription on the other hand seems to be specific for nutrient limitation-induced adaptations. Accordingly, genes containing STREs in their promoter region are induced upon heat shock, osmotic stress, high salt and ethanol treatments and glucose starvation, whereas genes containing the PDS element in their promoters are not induced under all of these conditions, but predominantly by glucose starvation. Interestingly in this context, it has been reported that trehalose accumulation in stationary phase is dependent on Rim15, since *rim15Δ* cells failed to accumulate trehalose when glucose was depleted from the medium. However, *rim15Δ* cells were not impaired in their ability to induce trehalose synthesis in response to heat shock (Reinders, 1998).

Although the mechanism by which trehalose synthesis is regulated in stationary phase and under heat shock conditions may be very different (Ref), these results support the suggestion that Rim15 is specifically involved in nutrient limitation-induced responses and to a lesser extend in general stress response. Various pathways have been implicated in stress signaling and some of them may indeed interact with the Ras/cAMP pathway. The nature of these interactions, however, are still unclear and will certainly have to be addressed in more detail in future studies.

With respect to the aim presented at the beginning, to unravel the mechanisms of how the Ras/cAMP pathway may regulate aspects of cell cycle and growth control, the identification of Gis1 does indeed add a new important element. Accordingly, our results suggest that the genes that are transcriptionally regulated by Gis1, and thereby most probably as well by Rim15, are involved specifically in adjusting cellular growth to nutrient conditions. Notably, overexpression of Gis1 was found to exacerbate the growth defect of mutants with attenuated PKA activity and to act moderately growth-inhibitory when overexpressed, suggesting that Gis1 controls a set of growth-inhibitory genes. This raises the question of which of the Gis1-controlled genes may directly be involved in cell proliferation control. Thus, the discovery of Gis1 may help to identify effectors of Ras/PKA/Rim15 that are specifically involved in growth control. Further analysis of Gis1-dependent transcription promises to yield valuable new insight in this topic. Thanks to a collaboration with Dr. H. Voshol, Novartis, we were already able to exploit one possibility to identify potential Gis1-regulated genes. Total protein extracts isolated from wild-type, *rim15Δ* and *gis1Δ* mutant cells grown to stationary phase were analyzed by 2D-gel analysis. This allowed the identification of gene products that showed altered expression levels or different post-translational modifications, as visualized by spot intensity and position on the gels. In a first step, we obtained sequences of proteins whose levels were either repressed or induced in the two mutant strains compared to the wild-type strain. Besides the identification of expected products of genes like *HSP12* and *HSP26*, another gene product, namely Gre1, drew our attention. Gre1 was completely absent in *rim15Δ* and *gis1Δ* mutants. In confirmation of our results presented on Gis1, the promoter of *GRE1* was found to contain six consensus PDS elements (Garay-Arroyo and Covarrubias, 1999). Not much is known about the biochemical function of *GRE1*, except that it belongs to a group of genes induced upon stress (*GRE1* for 'Genes de Respuesta a Estres', stress responsive genes, Spanish). However, we did not focus on further characterization of the Gre1 gene product, since it was reported that disruption of the *GRE* genes did not show a phenotype

in any of several stress conditions, including osmotic, ionic, oxidative and heat shock conditions (Garay-Arroyo and Covarrubias, 1999).

Taken together, one of the surprising aspects of our studies, was the finding that overexpression of *GIS1* strongly inhibited growth. This indicates that Gis1 stimulates the expression of growth-inhibitory genes and that PKA-mediated growth is at least partially mediated via Gis1. Even though the genes that are currently known to be under the control of Gis1 (*e.g. HSP12*, *HSP26*, *SSA3*, and *GRE1*) seem not to encode proteins that are directly related to growth, we have now the tools at hand to effectively search for such growth regulatory proteins. Accordingly, we may (i) carry out genetic screens for suppressors of the observed *GAL1-GIS1* induced growth arrest, (ii) apply more detailed 2-D gel analyses, and (iii) analyze the transcription profile of *gis1Δ* mutants using DNA microarray assays.

The second part of this work was dedicated to the identification of possible mechanisms of Rim15 activation. To date, the only post-translational regulatory mechanism known for Rim15 function is that its kinase activity is negatively regulated by PKA-dependent phosphorylation. Since overexpression of *RIM15* in exponentially growing cells only partially induced a starvation response (including trehalose synthesis, *SSA3*-induction and acquisition of thermotolerance). Rim15-activating factors, absent possibly in cells growing on glucose, may be necessary for full Rim15 activation. We have identified Bud14 as such a putative activating factor. *BUD14* was isolated in a screen for genes that, when overexpressed, exhibited Rim15-dependent activation of *SSA3* in exponentially growing cells. Interestingly, Bud14 contains a short sequence with similarity to the PP1 Glc7 recognition site. PP1 is the paradigm for the targeting subunit concept, where an unspecific catalytic subunit, Glc7, is targeted by several different regulatory subunits to a specific subcellular localization or process. Notably, Glc7 does not only play a key role in mitosis, but has previously also been suggested to interact with the Ras/cAMP pathway (Matsuura and Anraku, 1994; Frederick and Tatchell, 1996; Stark, 1996). Interestingly, our analyses show that Bud14 specifically interacts with Glc7 and that the Bud14/Glc7 holoenzyme is necessary for proper entry into stationary phase. Moreover, analyses of epistasis indicate that Bud14 interacts with the Ras/cAMP pathway, possibly at the level of Rim15.

The reversible phosphorylation of proteins regulates most aspects of cell life. The kinase activity of the protein kinase Rim15 is inhibited by phosphorylation, and it is very likely that removal of the corresponding phosphates is mediated via a specific phosphatase-mediated dephosphorylation process. A tantalizing model, in this context, would posit that Bud14

serves as regulatory subunit that mediates the interaction of Glc7 with the Ras/cAMP pathway through Rim15. Yet, based on our results, the underlying mechanism seems to be more complex than simple dephosphorylation-mediated activation of Rim15. Therefore, one focus of our future studies will be on the elucidation of the nature of the PP1-mediated effects on the Ras/cAMP. These studies will have to include analyses of the subcellular localization of Glc7, Bud14, and Rim15 by means of direct visualization of GFP-fusion proteins or by immunofluorescence studies of epitope-tagged proteins. In addition, we expect more detailed results by refined analyses of Rim15 kinase activity measurements and coimmunoprecipitation experiments. Knowledge on how protein phosphatases regulate signal transduction pathways is scarce and a direct involvement of Glc7 in the control of the Ras/cAMP pathway would undoubtedly be of great relevance for the general understanding of signal transduction pathways.

Another question to be addressed is how nutrient signals regulate the interaction of Bud14 with Glc7. Notably, other pathways may partially overlap with and/or act antagonistically to the Ras/cAMP pathway, including the TOR pathway and the glucose repression pathway. Based on picture that many signal transduction pathways are not linear, but highly interconnected between each other, it will be of particular interest to see whether PP1 integrates one of the already known nutrient signaling pathways with the Ras/cAMP/Rim15 pathway. As more results become available in the future, it should be easier to connect the single pieces and to draw a more complete picture. Conventional two-hybrid screens with Bud14, isolation of Bud14-TAP-tagged protein complexes, detailed microarray analysis of gene transcription in *bud14Δ* mutants, as well as further defined genetic screens will certainly allow us in the near future to identify the biochemical targets of the Bud14/Glc7 module and allow us to add an additional piece that may allow us to elucidate and/or entangle the mechanisms of nutrient signal transduction.

Chapter VI

Materials & Methods

Materials & Methods

Growth conditions, media, microbiological and recombinant DNA methods

Yeast and *Escherichia coli* media were prepared by standard recipes (Sambrook *et al.*, 1989; Rose *et al.*, 1990) unless otherwise stated. Yeast cells were grown at 30°C either in full YPD medium (1% w/v yeast extract, 2% 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 Kan^r selection, geneticin was added to a final concentration of 100 μg/ml. Solid medium contained 2% agar in addition. For experiments with stationary phase, cell cultures were grown on media containing only half the amount of carbon source (i.e. 1% glucose) to ensure this to be the limiting factor. For growth on other carbon sources than glucose, these were added to the media as indicated in the figure or table legends.

Bacterial cultures were grown at 37°C on LB medium (1% NaCl, 1% Bacto-tryptone, 0.5% yeast extract, pH 7.5). For Amp^r selection, ampicillin was added to a final concentration of 100 μg/ml. Solid medium contained 2% agar in addition.

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 were used (Sambrook *et al.*, 1989; Guthrie and Fink, 1991). Yeast transformations were performed using a modification of the Li^+-ion method (Gietz *et al.*, 1992). Sporulation experiments were performed essentially as described (Reinders *et al.*, 1998).

Materials used in Chapter II

Yeast strains

Table I. List of yeast strains used in Chapter II

Strain	Genotype		Source
YEF473	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	Bi and Pringle, (1996)
AR2	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rim15Δ::kanMX2/rim15Δ::kanMX2*	Reinders *et al.*, (1998)
SGY446	*MAT* α	*tpk1Δ::ADE8 tpk2-63^{ts} tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8*	Smith *et al.*, (1998)

The *S. cerevisiae* strains used in Chapter II are listed in Table I.

Bacterial strains

E. coli strain DB6656 (*pyrF79::Mu*) (Bach *et al.*, 1979) was used to rescue pYES-R based plasmids from strain AR2. The transformed cells were plated directly onto M9 minimal plates (Sambrook *et al.*, 1989) supplemented with 0.4% glucose, 4 μg/ml tryptophane and 100 μg/ml ampicillin.

Plasmid constructions

All plasmids used in Chapter II have been described elsewhere, as indicated in the text.

Materials used in Chapter III

Yeast strains

Table II. List of yeast strains used in Chapter III

Strain	Genotype		Source
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*	Reinders *et al.*, (1998)
AR1-1A	*MAT* **a**	*his3 leu2 lys2 trp1 ura3*	segregant from AR1
AR1-1C	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2*	segregant from AR1
AR2	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rim15Δ::kanMX2/rim15Δ::kanMX2*	Reinders *et al.*, (1998)
OL86	*MAT* α	*leu2 trp1 ade2 cdc25-5*	Camonis *et al.*, (1986)
PD6517	*MAT* α	*ade8 leu2 trp1 cdc35-10*	dos Passos *et al.*, (1992)
SGY446	*MAT* α	*tpk1Δ::ADE8 tpk2-63*(Ts) *tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8*	Smith *et al.*, (1998)
ASY18	*MAT* α	*tpk1Δ::ADE8 TPK2 tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8*	Smith *et al.*, (1998)
NB19	*MAT* α	*leu2 trp1 ade2 cdc25-5 gis1Δ::kanMX2*	this study
NB21	*MAT* α	*ade8 leu2 trp1 cdc35-10 gis1Δ::kanMX2*	this study
NB23	*MAT* α	*tpk1Δ::ADE8 tpk2-63*(Ts) *tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8 gis1Δ::kanMX2*	this study
IP6	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 gis1Δ::HIS3/GIS1 rim15Δ::kanMX2/RIM15*	this study
CDV100	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 gis1Δ::HIS3/GIS1 rim15Δ::kanMX2/RIM15 rph1Δ::TRP1/RPH1*	this study
CDV100-10D	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 gis1Δ::HIS3*	segregant from CDV100
CDV100-4D	*MAT* α	*his3 leu2 lys2 trp1 ura3 gis1Δ::HIS3*	segregant from CDV100
CDV100-6C	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 gis1Δ::HIS3 rim15Δ::kanMX2*	segregant from CDV100

Table II. continued

Strain	Genotype		Source
CDV100-10A	*MAT* α	*his3 leu2 lys2 trp1 ura3 gis1Δ::HIS3 rim15Δ::kanMX2*	segregant from CDV100
CDV101	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 gis1Δ::HIS3/gis1Δ::HIS3*	CDV100-10D × CDV100-4D
CDV104	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 gis1Δ::HIS3/gis1Δ::HIS3 rim15Δ::kanMX2/ rim15Δ::kanMX2*	CDV100-6C × CDV100-10A
NB14	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 GIS1-Myc13:TRP1*	this study
NB15	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2 GIS1-Myc13:TRP1*	this study
W303-1A	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Thomas and Rothstein, (1989)
Wmsn2msn4	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 msn2-Δ3::HIS3 msn4-1::TRP1*	Martínez-Pastor *et al.*,(1996)
CDV120	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ*	this study
CDV121	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ rim15Δ::kanMX2*	this study
CDV122	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ gis1Δ::kanMX2*	this study
CDV123	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ msn2-Δ3::HIS3 msn4-1::TRP1*	this study
CDV124	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ msn2-Δ3::HIS3 msn4-1::TRP1 gis1Δ::kanMX2*	this study
IP17	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ rph1Δ::TRP1*	this study
CDV125	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ*	this study
CDV126	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ rim15Δ::kanMX2*	this study
CDV127	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ gis1Δ::kanMX2*	this study

Table II. continued

Strain	Genotype		Source
CDV128	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ msn2-Δ3::HIS3 msn4-1::TRP1*	this study
CDV129	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ msn2-Δ3::HIS3 msn4-1::TRP1 gis1Δ::kanMX2*	this study
IP13	*MAT* **a**	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ rph1Δ::TRP1*	this study

The *S. cerevisiae* strains used in Chapter III are listed in Table II. To construct *gis1Δ*, *rph1Δ*, and *rim15Δ* mutants, the complete *GIS1, RPH1*, and *RIM15* coding regions were deleted by the PCR method (Baudin *et al.*, 1993) using the Expand™ High Fidelity PCR System (Roche Diagnostics GmbH) and either plasmid pRS303 (*HIS3*, Sikorski and Hieter, 1989), pRS304 (*TRP1*, Sikorski and Hieter, 1989), or pFA6a (*kanMX2*, Wach *et al.*, 1994) as template. The PCR products that contained flanking sequences of *GIS1*, *RPH1,* and *RIM15* separated by the *HIS3, TRP1,* or *kanMX2* modules were extracted with phenol/chloroform, precipitated, and used for transformations. The *gis1Δ::HIS3* deletion cassette was transformed into strain AR2 to construct IP6; the *gis1Δ::kanMX2* deletion cassette was transformed into strains OL86, PD6517, and SGY446 to construct NB19, NB21, and NB23, respectively; and the *rph1Δ::TRP1* deletion cassette was transformed into strain IP6 to construct CDV100. Strains CDV101 and CDV104 were constructed by crossing haploid segregants from CDV100 as follows: CDV100-10D × CDV100-4D (CDV101) and CDV100-6C × CDV100-10A (CDV104).

Linearized, *Nco*I-cut integrative vectors pCTT1-18/7x and pLS9-PDS were transformed into strain W303-1A to construct CDV120 and CDV125, respectively, co-transformed with the *rim15Δ::kanMX2* deletion cassette into strain W303-1A to construct CDV121 and CDV126, respectively, co-transformed with the *gis1Δ::kanMX2* deletion cassette into strain W303-1A to construct CDV122 and CDV127, respectively, transformed into strain Wmsn2msn4 to construct CDV123 and CDV128, respectively, co-transformed with the *gis1Δ::kanMX2* deletion cassette into strain Wmsn2msn4 to construct CDV124 and CDV129, and co-

transformed with the *rph1Δ::TRP1* deletion cassette into strain W303-1A to construct IP17 and IP13, respectively.

Transformants that had *GIS1* replaced by *gis1Δ::HIS3* or *gis1Δ::kanMX2*, *RPH1* replaced by *rph1Δ::TRP1*, or *RIM15* replaced by *rim15Δ::kanMX2* were confirmed by PCR and/or Southern blot analysis (data not shown). NB14 and NB15 were generated by PCR-based, chromosomal *Myc13*-tagging of *GIS1* in AR1-1A and AR1-1C, respectively, using pFA6a-13Myc-TRP1 as template (Longtine *et al.*, 1998).

Plasmid constructions

For construction of the galactose-inducible *GAL1-GIS1* and *GAL1-RPH1* alleles, the full length *GIS1* and *RPH1* coding sequences were amplified using the Expand Long Template PCR System (Roche Diagnostics GmbH) and genomic DNA of strain AR2 (Table II) as template. *Sal*I restriction sites were introduced immediately upstream of both ATG start codons and *Not*I restriction sites were introduced 227 bp and 220 bp downstream of the *GIS1* and *RPH1* stop codons, respectively. The PCR products were cloned at the *Sal*I-*Not*I sites of YCpIF2 (Foreman and Davis, 1994) to yield YCpIF2-*GIS1* and YCpIF2-*RPH1*. Plasmids YCp*ADH1-GIS1* and YCp*ADH1-RPH1* were constructed by replacing the *GAL1* promoter-containing *Apa*I-*Sal*I fragments of YCpIF2-*GIS1* and YCpIF2-*RPH1*, respectively, with a PCR-generated *Apa*I-*Sal*I fragment containing the 854 nucleotides upstream of and including the *ADH1* start codon. Plasmids YCpIF2-*RIM15*, YCp*ADH1*, and YCp*ADH1-RIM15* were described earlier (Reinders *et al.*, 1998). To fuse Gis1 and Rph1 to the LexA DNA-binding domain (DBD) coding sequence in plasmid pEG202 (Zervos *et al.*, 1993), *GIS1* and *RPH1* full-length coding sequences were amplified as described above and cloned at the *Sal*I-*Not*I sites of a modified version of plasmid pEG202 that contains a unique *Sal*I site in its polylinker. The constructs contain 4 (EFVD) additional amino acids between the LexA DBD and the first amino acid (M) of the fused protein. Plasmid pLS9-PDS was constructed by cloning a synthetic double stranded oligonuceotide consisting of 37 bp identical to the *SSA3*-PDS region (-206 through –170), plus AATT overhangs and an additional *Hind*III restriction site upstream of the *SSA3*-PDS region, into the *Eco*RI site of pLS9 (Sarokin and Carlson, 1986). Plasmid pCTT1-18/7 has been described (Marchler *et al.*, 1993).

Plasmid pLS9-*GRE1* was constructed by cloning a PCR-generated *Eco*RI fragment containing the 778 nucleotides upstream of the *GRE1* start codon (using genomic DNA of strain AR2 as template) into the *Eco*RI site of pLS9 (Sarokin and Carlson, 1986). Correct orientation of integration was confirmed by subsequent sequencing. Plasmid YEplac181-*GRE1-GFP* was constructed by cloning simultaneously a PCR-generated *EcoRI*/*Xba*I fragment containing the 778 nucleotides upstream of the *GRE1* (see above) and a PCR-generated *Xba*I/*Pst*I fragment (using plasmid pGNG1 (Mo Bi Tec GmbH) as a template) containing the coding sequence for *GFP* including 24 bp upstream of the ATG start codon of *GFP* and 25bp downstream of the corresponding stop codon into the *Eco*RI-*Pst*I site of YEplac181 (Gietz and Sugino, 1988).

Materials used in Chapter IV

Yeast strains

Table III. List of yeast strains used in Chapter IV

Strain	Genotype		Source
YEF473	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	Bi and Pringle, (1996)
AR1-1A	*MAT* **a**	*his3 leu2 lys2 trp1 ura3*	Reinders *et al.*, (1998)
AR1-1C	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2*	Reinders *et al.*, (1998)
EGY48	*MAT* α	*his3 trp1 ura3 LEU2::pLexAop6-LEU2*	Zervos *et al.*, (1993)
KT1960	*MAT* α	*ura3-52 leu2 his3 trp1*	K. Tatchell
KT1961	*MAT* **a**	*ura3-52 leu2 his3 trp1*	K. Tatchell
KT1703	*MAT* α	*ura3-52 leu2 his3 trp1 glc7-132*	K. Tatchell
KT1706	*MAT* α	*ura3-52 leu2 his3 trp1 glc7-133*	K. Tatchell
KT1708	*MAT* α	*ura3-52 leu2 his3 trp1 glc7-127*	K. Tatchell
CDV158	*MAT* **a**	*ura3-52 leu2 his3 trp1 bud14Δ::kanMX2*	this study
IP31	*MAT* α	*ura3-52 leu2 his3 trp1 rim15Δ::kanMX2*	this study
IP36	*MAT* **a**/α	*ura3-52/ura3-52 leu2/leu2 his3/his3 trp1/trp1 BUD14/bud14Δ::kanMX2 RIM15/rim15Δ::kanMX2*	CDV158 × IP31
IP36-1A	*MAT* **a**	*ura3-52 leu2 his3 trp1 bud14Δ::kanMX2 rim15Δ::kanMX2*	segregant from IP36
IP36-1D	*MAT* α	*ura3-52 leu2 his3 trp1 bud14Δ::kanMX2*	segregant from IP36

Table III. continued

Strain	Genotype		Source
CDV147	*MAT* **a**/α	*ura3-52/ura3-52 leu2/leu2 his3/his3 trp1/trp1*	KT1960 × KT1961
IP38	*MAT* **a**/α	*ura3-52/ura3-52 leu2/leu2 his3/his3 trp1/trp1 bud14Δ::kanMX2/bud14Δ::kanMX2*	CDV158 × IP36-1D
IP19	*MAT* **a**	*ura3-52 leu2 his3 trp1 BUD14-Myc13:kanMX6*	this study
CDV132	*MAT* **a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 BUD14-Myc13:kanMX6/BUD14*	this study
CDV132-1C	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 BUD14-Myc13:kanMX6*	segregant from CDV132
CDV138	*MAT* **a**	*his3 leu2 lys2 trp1 ura3 BUD14-Myc13:kanMX6 GLC7-HA:TRP1*	this study
ASY18	*MAT* α	*tpk1Δ::ADE8 TPK2 tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8*	Smith *et al.*, (1998)
PE12	*MAT* α	*tpk1Δ::ADE8 TPK2 tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8 bud14Δ::kanMX2*	this study
SGY446	*MAT* α	*tpk1Δ::ADE8 tpk2-63^{ts} tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8*	Smith *et al.*, (1998)
PE15	*MAT* α	*tpk1Δ::ADE8 tpk2-63^{ts} tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8 bud14Δ::kanMX2*	this study
OL86	*MAT* α	*leu2 trp1 ade2 cdc25-5*	Camonis *et al.*, (1986)
PD6517	*MAT* α	*ade8 leu2 trp1 cdc35-10*	dos Passos *et al.*, (1992)

The *S. cerevisiae* strains used in Chapter IV are listed in Table III. To construct *bud14Δ* and *rim15Δ* mutants, the complete *BUD14* and *RIM15* coding regions were deleted by the PCR method (Baudin *et al.*, 1993) using the Expand™ High Fidelity PCR System (Roche Diagnostics GmbH) and plasmid pFA6a-*kanMX2* (Wach *et al.*, 1994) as template. The PCR products that contained flanking sequences of *BUD14* and *RIM15* separated by the *kanMX2* module were extracted with phenol/chloroform, precipitated, and used for transformations. The *bud14Δ::kanMX2* deletion cassette was transformed into strains KT1961, ASY18, and SGY446 to construct CDV158, PE12, and PE15, respectively, and the *rim15Δ::kanMX2* deletion cassette was transformed into strain KT1960 to construct IP31. Transformants that

had *BUD14* replaced by *bud14Δ::kanMX2* or *RIM15* replaced by *rim15Δ::kanMX2* were confirmed by PCR.

Strain IP36 was constructed by crossing CDV158 × IP31. Strains CDV147 and IP38 were obtained by crossing KT1960 × KT1960 and CDV158 × IP36-1D, a haploid segregant of IP36, respectively.

IP19 and CDV132 were generated by PCR-based, chromosomal *Myc13*-tagging of *BUD14* in KT1961 and YEF473, respectively, using pFA6a-*13Myc-kanMX6* as template (Longtine *et al.*, 1998). CDV138 was generated by PCR-based, chromosomal HA-tagging of *GLC7* in CDV132-1C, a haploid segregant of CDV132.

Bacterial strains

E. coli strain DB6656 (*pyrF79::Mu*) (Bach *et al.*, 1979) was used to rescue pSEY18 based plasmids from strain AR1-1A as described above

Plasmid constructions

To fuse Glc7 and its various point mutations to the LexA DNA-binding domain (DBD) coding sequence in plasmid pEG202 (Zervos *et al.*, 1993), *GLC7, glc7-127, glc7-132* and *glc7-133* full-length coding sequences were amplified using the Expand Long Template PCR System (Roche Diagnostics GmbH) and genomic DNA of strains KT1960, KT1703, KT1706 and KT1708 (Table I), respectively, as template. *Nco*I restriction sites were introduced immediately upstream of the ATG start codon and *Xho*I restriction sites were introduced 220 bp downstream of the *GLC7* stop codon. The PCR products were cloned at the *Nco*I-*Xho*I sites of pEG202. The constructs contain 9 (EFPGIRRPW) additional amino acids between the LexA DBD and the first amino acid (M) of the fused proteins. To fuse Bud14 to the LexA DBD, *BUD14* full-length coding sequence was amplified by PCR using genomic DNA of strain KT1960 as template. *Eco*RI restriction sites were introduced immediately upstream of the ATG start codon and *Xho*I restriction sites were introduced 292 bp downstream of the *BUD14* stop codon. The PCR product was cloned at the *Eco*RI-*Xho*I sites of pEG202. The construct contains 2 (EF) additional amino acids between the LexA DBD and the first amino acid (M) of the fused protein. For construction of a fusion of Bud14 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. DeMarini, unpublished), *BUD14* full-length coding sequence was amplified as described above and cloned at the *EcoR*I-*Xho*I sites of pJG4-5. To obtain fusions of the Bud14^{V377A} and Bud14^{F379A} mutant proteins to the AD of pJG4-5, point mutations were constructed with the QuickChange Site-Directed mutagenesis Kit (Stratagene) using appropriate primers that introduced the corresponding mutations and pJG4-5-*BUD14* as template. All mutations introduced were confirmed by subsequent sequencing. For construction of a fusion of Rim15 to the AD of pJG4-5, an *Sal*I-*Not*I fragment was isolated from YCpIF2-*RIM15* (Reinders *et al.*, 1998) and cloned at the *Xho*I-*Not*I sites of pJG4-5. As a negative control, *MSB2* was fused to the DBD in pEG202 and the AD in pJG4-5 as described earlier (Simon *et al.*, 1995).

To obtain HA-epitope tagged versions of Glc7 and its various point mutations, *GLC7, glc7-127, glc7-132* and *glc7-133* full-length coding sequences were amplified by PCR using genomic DNA of strains KT1960, KT1703, KT1706 and KT1708, respectively, as template. *Pst*I restriction sites were introduced immediately upstream of the ATG start codon and *Hind*III restriction sites were introduced 151 bp downstream of the *GLC7* stop codon. The PCR products were cloned at the *Pst*I-*Hind*III sites of YCpHA22. YCpHA22 is a YCplac22 (Gietz and Sugino, 1988) derivative, that was constructed by cloning a *EcoR*I-*Hind*III fragment containing the yeast *GAL1* promoter and a double HA tag from pAS24 (YCp111::*GAL1*-HA; gift of M.N. Hall, Biocenter, Basel) at the *EcoR*I-*Hind*III sites of YCplac22. pAS24 is YCplac111 (Gietz and Sugino, 1988). To obtain a GST-epitope tagged version of Bud14, *BUD14* full-length coding sequence was amplified by PCR using genomic DNA of strain KT1960 as template. A *Hind*III restriction site was introduced immediately upstream of the ATG start codon and a *Sac*I restriction site was introduced 297 bp downstream of the *BUD14* stop codon. The PCR product was cloned at the *Hind*III-*Sac*I sites of YCpIF2-*GST* (Reinders *et al.*, 1998). To obtain *GST*-epitope tagged versions of the Bud14^{V377A} and Bud14^{F379A} mutant proteins, point mutations were generated as described above using YCpIF2-*GST-BUD14* as a template.

To express *BUD14* under its own promoter from high-copy number plasmids, *BUD14* full-length coding sequence was amplified by PCR using genomic DNA of strain KT1960 as template. A *Sph*I restriction site was introduced 681 bp upstream of the ATG start codon containing the *BUD14* promoter and the full length *BUD14* gene and an *EcoR*I restriction sites was introduced 282 bp downstream of the *BUD14* stop codon. The PCR product was

cloned at the *Sph*I-*Eco*RI sites of YEplac181 (Gietz and Sugino, 1988). To obtain corresponding *bud14*^{V377A} and *bud14*^{F379A} mutant genes in the same expression vector, point mutations were generated as described above using YEplac181-*BUD14* as a template.

pHAC195-*GST-RIM15* expresses a double-tagged Rim15 protein (with a N- and C-terminal GST and HA fusion, respectively) under the control of *GAL1*. It was constructed by the yeast-based, oligonucleotide-mediated gap repair technique (DeMarini *et al.*, 2001), using plasmid pHAC195 as target vector, YCpIF2-*GST-RIM15* as substrate DNA, and a pair of appropriate oligonucleotides that mediate correct integration of *GST-RIM15* into pHAC195 (C. De Virgilio, unpublished). pHAC195 is a YEplac195 derivative (Gietz and Sugino, 1988) containing a 550 bp *Sph*I-*Hind*III fragment that encodes a triple HA-tag followed by a *CYC1* terminator sequence. Plasmid pHAC195-*GST-RIM15*^{K823Y} was constructed by generating a point mutation by site-directed mutagenesis essentially as described above using pHAC195-*GST-RIM15* as template.

Methods

DNA sequencing and sequence analysis

DNA sequences were obtained using the BigDye™ Primer cycle sequencing Kit and an automated sequencer ABI 301 (both from Applied Biosystems) according to the manufacturer's instructions.

Enzyme assays and determination of metabolite levels

Glucose concentrations in media were determined using an enzymatic glucose assay with GOD (GLU, Roche Diagnostics GmbH) according the manufacturer's instructions. For determination of trehalose concentrations, 10 ml exponentially growing cells or 1 ml stationary-phase cells were filtered (Whatman GF/C), washed four times with 5 ml distilled H_2O, resuspended in 1 ml H_2O and transferred to a boiling water bath for 5 min. After centrifugation (5 min. at 15'000 g), 50 μl of the supernatant was incubated with 0.01 units of trehalase (EC 3.2.1.28) (Sigma) in 50 μl 0.2M Na-acetate, 30 mM $CaCl_2$, pH 5.7 at 37°C for

at least 2 hrs, cleaving one moiety trehalose into 2 moieties glucose. The resulting glucose was determined as described above. Control values were obtained by incubation of samples without trehalase. These values, representing contaminating glucose carried over from the medium during the extraction procedure, were subtracted from the corresponding trehalase treated samples. β-Galactosidase activity was assayed using *o*-nitrophenyl-β-D-galactoside as substrate (Miller, 1972).

Preparation and Northern analysis of RNA

Extraction of total cellular RNA was performed as described previously (Piper, 1994). For Northern analysis, 10 µg of total RNA was separated on 1.1% agarose gels containing 0.65 M formaldehyde, transferred to nitrocellulose membranes (Protran BA 83, 0.2µm; Schleicher and Schuell, Germany) in 20X SSC, and hybridized with [^{32}P]dCTP labeled DNA fragments that were amplified by PCR from genomic DNA (of strain AR2; Table I) and labeled by using the Prime-It Random Primer Labeling Kit (Stratagene). The primers used for PCR to generate *SSA3*, *HSP12*, *HSP26*, and *SSB1* DNA fragments have been described (Reinders *et al.*, 1998), the ones to generate the 2.78 kb *TPS2* DNA fragment were as follows: 5'-ACCACTGCCCCG GGCAATTCTCCAAAGAAGAGACAG-3' (forward) and 5'-ACTATTATATATATGCCCG GGTCGCAACACCACATA-3' (reverse).

Quantification of proteins

Protein concentrations were either measured by the Bio-Rad protein assay according to the manufacturer's instructions or by a modified Lowry assay (Peterson, 1977) using BSA as standard.

Immunoblot analysis

For immunodetection of epitope-tagged proteins, total cellular proteins were extracted by vortexing cells in lysis buffer [0.2 M Tricine [Na^+], pH 7.0, and one tablet of Complete Protease Inhibitor Cocktail (Roche Diagnostics GmbH) per 50 ml] in the presence of acid-

washed glass-beads (0.4-mm diameter; Merck), run on SDS-polyacrylamide gels (10%, w/v, acrylamide), and transferred electrophoretically to nitrocellulose membranes as described previously (Reinders *et al.*, 1999). Blots were stained with Ponceau S to ensure equal protein loading. The membranes were incubated with either monoclonal mouse anti-myc ($MIgG_1$) antibodies (diluted 1:5000, Invitrogen), monoclonal mouse anti-GST (GST [B-14]) antibodies (diluted 1:2000, Santa Cruz Biotechnology, Inc.), or monoclonal mouse anti-HA antibodies (diluted 1:2000, gift of H.-P. Hauri, Biocenter, Basel), followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG secondary antibodies (Biosource, USA). Reactive bands were visualized by an enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Fluka, Switzerland). Alternatively, horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibodies (Biorad) were used and reactive bands were detected using the ECL Western blotting detection reagents (Amersham pharmacia biotech) according the manufacturer's instructions. The bands were visualized by autoradiography on a BioMax MR film (Kodak) and developed using an automated film processor (M35 X-OMAT, Kodak).

Two-hybrid analyses

Interactions of proteins were tested by two-hybrid analysis (Fields and Sternglanz, 1994) using the LexA system described in detail elsewhere (Gyuris *et al.*, 1993). Strain EGY48 (Table III) 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 pJG4-5 or a pJG4-5-derived plasmid expressing an AD fusion protein. Plasmids were constructed as described above. β-galactosidase activities were then assessed in three independent clones of each strain grown for 16 h at 30°C in minimal medium containing 2% w/v galactose, 1% w/v raffinose, and 20 μg/ml leucine.

Co-precipitation experiments

To perform co-precipitation experiments between Bud14 and wild-type and mutant Glc7 proteins, strain IP19, expressing a chromosomally integrated myc-tagged Bud14 protein was transformed with either YCpHA22, YCpHA22-*GLC7*, YCpHA22-*glc7-127*, YCpHA22-*glc7-*

132, or YCpHA22-*glc7-133*. To perform co-precipitation experiments between Glc7 and wild-type and mutant Bud14 proteins, strain KT1961 was transformed with YCpHA22-*GLC7* and co-transformed with either YCpIF2-GST, YCpIF2-GST-*BUD14*, YCpIF2-GST-*bud14*^{V377A} or YCpIF2-GST-*bud14*^{F379A}. Overnight cultures grown in SD medium with 2% w/v raffinose were diluted to an OD_{600} of 0.4 in the same medium, incubated for additional 2 hrs at 30°C, after which the cultures were supplemented with 4% w/v galactose to induce *GAL1*-driven expression of the Glc7 and Bud14 proteins during 4 additional hours at 30°C. Cells were harvested by centrifugation (10 min. at 4'000g) and resuspended in ice-cold lysis buffer (50 mM Tris-HCl at pH 7.5, 0.1 M NaCl, 1 mM EDTA, 1% NP-40, and one tablet of Complete Protease Inhibitor cocktail [Roche Diagnostics GmbH] per 50 ml) in the presence of acid-washed glass beads (0.4-mm diameter; Merck). The cells were disrupted by 4 cycles of 30 seconds in a cell disruptor (FastPrep FP120, BIO101/Savant). The extracts were then clarified three times by centrifugation at 4°C in a microfuge at 17'000 rpm, 10 min each time.

The GST-fusions were purified from clarified cell extracts after a 4-hr incubation at 4°C with glutathione-Sepharose™ 4B beads (50 µg/ml; Pharmacia). Protein-bound beads were pelleted and washed four times with lysis buffer removing the supernatants each time completely with a Hamilton syringe. Pellets were resuspended in 50 µl SDS-gel loading buffer and boiled for 5 min. From each sample an aliquot of 10 µl was loaded onto two separate SDS-gels (10%, Laemmli, 1970). Immunoblot analysis was performed as described above.

The HA or myc-tagged fusion proteins were purified from clarified cell extracts with the corresponding antibodies and protein G-agarose (Immunoprecipitation Kit [Protein G]; Roche Diagnostics GmbH) following the manufacturer's instructions. Further handling of the samples has been outlined above.

Protein kinase assays

GST-Rim15 and GST-Rim15^{K823Y} were expressed in wild-type and *bud14Δ* cells from the *GAL1* promoter. Overnight cultures in SD medium with 2% w/v raffinose were diluted to an OD_{600} of 0.4 in the same medium, incubated for 2 hrs at 30°C followed by a 4 hrs incubation at 30°C after the addition of 4% w/v galactose to induce *GAL1*-driven expression. Cells were harvested by centrifugation (10 min at 4'000g) and resuspended in ice-cold lysis buffer (50 mM Tris-HCl at pH 7.5, 0.1 M NaCl, 1 mM EDTA, 1% NP-40, and one tablet of Complete

Protease Inhibitor cocktail [Roche Diagnostics GmbH] per 50 ml) in the presence of acid-washed glass beads (0.4-mm diameter; Merck). The cells were disrupted by 4 cycles of 30 seconds in a cell disruptor (FastPrep FP120, BIO101/Savant). The extracts were clarified three times by centrifugation at 4°C in a microfuge at 17'000 rpm, 10 min each time. The GST-fusions were purified from clarified cell extracts after a 4-hr incubation at 4°C with glutathione-Sepharose™ 4B beads (50 μg/ml; Pharmacia). Protein-bound beads were pelleted, washed four times with lysis buffer and eluted by a 1-hr incubation at 4°C in kinase buffer [50mM Tris-HCl at pH 7.5, 20 mM $MgCl_2$, 1mM DTT, and 1 mM ATP] containing 15mM L-glutathione reduced (Fluka). Kinase assays were performed at 30°C for 20 min in reaction buffer (kinase buffer containing 10 μCi [γ-^{32}P]ATP and 250 μg/ml α-casein, and, where indicated, different concentrations of PP1 inhibitor I-1 [Promega[). Additionally, where indicated, the whole extraction procedure and the kinase reaction were performed in the presence of a phosphatase inhibitor mix (50 mM NaF, 10mM Na-orthovanadate, 15 mM p-NO_2-phenylphosphate, 50 mM β-glycerophosphate, and 5 mM Na-pyrophosphate). Reactions were stopped by adding 5 μl SDS-gel loading buffer and subsequent boiling for 5 min. Samples were subjected to SDS-PAGE, and the gels were then dried and exposed to X-ray film.

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