Instituto Juan March
de Estudios e Investigaciones

35 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Signal Transduction Pathways Essential for Yeast Morphogenesis and Cell Integrity

Organized by

M. Snyder and C. Nombela

G. Ammerer
J. Ariño
K. J. Blumer
S. Brown
H. Bussey
D. Drubin
A. Durán
B. Errede
G. R. Fink
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C. Mann
C. Nombela
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INTRODUCTION

M. Snyder and C. Nombela

SIGNAL TRANSDUCTION PATHWAYS ESSENTIAL FOR YEAST MORPHOGENESIS AND CELL INTEGRITY

Madrid (Spain). 28-30 November, 1994.

The mechanism by which cells form particular shapes and division patterns, and the manner by which they control their response to extracellular conditions are issues of fundamental importance to living organisms. Cells form specialized shapes to carry out specific cellular processes, and cortical intracellular and extracellular components are essential for (i) forming and maintaining cell shape (ii) maintaining cell integrity under a variety of different environmental stress conditions and (iii) sensing and transmitting extracellular signals.

Yeast cells undergo specialized morphogenic processes which depend upon the extracellular conditions. In the presence of ample nutrients, they grow vegetatively by budding. During nutrient limiting conditions they undergo pseudohyphal growth, and in the presence of pheromone, they form shmoos. The control of morphogenesis and cell integrity during growth of yeast cells is mediated by a complex set of functions such as the synthesis of components of the external cell envelope, the vectorial process of polarized growth, and the regulation of the mitotic cell cycle.

The synthesis of an elaborate cell wall is important not only for appropriate morphogenic processes, but also for maintaining osmotic stability when cells are exposed to significant changes in the osmotic strength of the outside medium.

This workshop has been devoted to the analysis of signaling pathways important for the regulation of for cell morphogenesis and cell wall integrity. In the last few years considerable progress has been made toward the elucidation of basic mechanisms important for these processes. Of particular importance has been the unraveling of protein phosphorylation and GTPase pathways which participate in morphogenesis and cell integrity maintenance. The components of three different MAP kinase pathways have been elucidated: one that is controlled by protein kinase C functions the maintenance of cell integrity, another functions in the regulation of the response to osmotic stress conditions, and the third functions in both pseudohyphal formation and signal transduction during mating. Different GTP binding proteins that function in the mating signal

transduction pathway and in bud site selection and bud formation have been identified.

The purpose of this workshop was to bring together researchers involved in different aspects of cell signaling mechanisms important for cell morphogenesis and cell integrity. Signal transduction cascades which are important for vegetative growth, pseudohyphal growth, osmoregulation and mating were discussed, and factors which affect signaling mechanisms, adaptation, and the control of cell division was reported. Finally, how different signaling pathways participate in steps polarized cell growth and cell wall synthesis was presented. Recent contributions toward our understanding in these areas are presented in the following abstracts.

Michael Snyder
César Nombela

I. Mating Signalling Pathways

Fus3 dependent protein phosphorylation and the pheromone response

Gustav Ammerer Department of Biochemistry and Molecular Cell Biology, University of Vienna, Austria

Two proteins have been identified that mediate a haploid cells specific responses to pheromone: 1) Ste12, the major DNA binding factor responsible for the transcription of conjugation specific genes. 2) Far1, a protein involved in the negative regulation of G1 cyclins and thus responsible for pheromone dependent cell cycle arrest. Since it is generally assumed that these two proteins are *in vivo* substrates of the MAP kinase Fus3 we tried to define how protein phosphorylation might effect their function.

Using *in vivo* footprinting methods we found that a functional *FUS3* or *KSS1* gene is necessary to detect Ste12 bound at the pheromone inducible *FUS1* promoter. In cells lacking both of these MAP kinases the promoter is not occupied even if Ste12 protein is present at normal levels and can be found in the nucleus. However, in *fus3 kss1* double mutants, Ste12 appears to be phosphorylated only to a minor extent. This is in contrast to normal but still uninduced wildtype cells where Ste12 is highly phosphorylated. Although under these conditions most of the Ste12 binding sites appear to be occupied, the promoter is barely active compared to fully induced conditions. Surprisingly, pheromone induction will only lead to a small increase in total Ste12 phosphorylation. We thus conclude that Fus3 primarily regulates DNA binding of Ste12. However, a second phosphorylation event, requiring a high concentration of active kinase might still be necessary for the full induction of the transcriptional response. Since Ste12 phosphorylation does not increase correspondingly with the activity of the kinase we cannot exclude yet that transcription factors other than Ste12 are important targets for Fus3.

Reportedly, Fus3 dependent phosphorylation of Far1 correlates very well with Far1's ability to cause G1-arrest. Nevertheless, it has remained unclear whether this phosphorylation of Far1 is functionally relevant. We tried to prove the point by identifying and mutating potential phosphorylation sites in Far1. Substitution mutations were obtained at most of the SP motifs located within the domains shown to be important for cell cycle arrest. Although some of the substitutions clearly affected the Fus3 dependent phosphorylation pattern none of them abolished the G1 arrest function of Far1. Similar results were obtained with *FAR1* constructs expressing multiple substitutions, although in all cases some Fus3 dependent and pheromone inducible phosphorylation could still be observed. We conclude that either Fus3 recognizes sequences other than normal MAP kinase consensus sites or another (but still Fus3 dependent) protein kinase mediates pheromone induced phosphorylation of Far1. Furthermore, it remains unclear how significant Far1 phosphorylation is for G1 specific cell cycle arrest.

Control of Adaptation to Mating Pheromone by G Protein β Subunits. (A.V. Grishin and K.J. Blumer. Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110)

Yeast cells use several mechanisms to recover from mating pheromone-induced G1 arrest, including pheromone degradation, receptor desensitization and endocytosis, and *SST2*-mediated desensitization. Recovery (desensitization or adaptation) is also controlled by G protein β subunits (*STE4* gene product) involved in pheromone response, because cells expressing G_β subunits that are not phosphorylated display a moderate supersensitive phenotype and recover more slowly from pheromone-induced G1 arrest.

To determine how G_β subunits promote adaptation, we examined the phenotypes of wild type cells overexpressing mutant G_β subunits (encoded by $STE4^{sd}$ alleles) that are specifically defective for their ability to activate the mating pheromone response pathway. These cells respond to pheromone with nearly normal efficiency, as judged by quantitative mating, transcriptional, and cell cycle arrest assays. However, after cells arrest in G1, they rapidly recover from pheromone action and resume proliferation. Genetic tests indicate that this hyperadaptive phenotype does not involve pheromone proteolysis, receptor desensitization, or *SST2* function. However, hyperadaptation does require the phosphorylation domain of the mutant G_β subunits. Accordingly, G_β subunits may transduce two distinct signals, one that causes pheromone response, and another that promotes adaptation.

To identify components of a putative G_β-mediated adaptation pathway, we isolated mutants that fail to hyperadapt when they overexpress $STE4^{sd}$ alleles. Two genes were identified: *MCM1*, which encodes the yeast serum response factor homolog that is involved in several processes including in cell type determination, and *BSA1*, which encodes a previously unidentified Zn-finger protein.

Currently, we are searching for genes that function in concert with Mcm1p and Bsa1p in an adaptation signaling pathway triggered by G_β subunits. One candidate is *CLN3*, which contains high affinity Mcm1p binding sites in its promoter. Consistent with this possibility, *cln3* mutants do not hyperadapt when they overexpress $STE4^{sd}$ alleles. We have also tested whether several other genes that may affect G1 progression are required for G_β-mediated hyperadaptation. Whereas *cln1, cln2, hcs26* or *orfD* mutations did not prevent hyperadaptation, a *pho85* mutation did. We speculate that G_β subunits may transmit an adaptive signal that induces the activity or expression Cdc28-Cln3 and/or Pho85-cyclin complexes.

Coordination and Fidelity of Signal Transmission in Separate MAPK Activation Pathways

B. Errede[1], B. Yashar[1], K. Irie[2], K, Matsumoto[2], R. Cade[1], Y. Komada[3], and D.E. Levin[3]

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We presently know of four separate but structurally related mitogen activated protein kinase (MAPK) activation pathways that mediate distinct cellular responses in *S. cerevisiae* [see (1) and (2) for review and references therein]. Two of these are the focus of our work. One pathway controls the response to mating pheromones. Pheromone-receptor binding informs cells of the proximity of a mating partner and induces differentiation to a mating competent state. Signal transmission is mediated by a G-protein that, by an as yet unknown mechanism, stimulates a phosphorylation cascade which is conserved from yeast to mammals. Ste11 (*Ste=Sterile*), a MEK-kinase (MEKK), phosphorylates and activates Ste7, a MAPK/ERK-kinase (MEK). Ste7 in turn phosphorylates and activates the redundant MAPK related Fus3 and Kss1 enzymes. The other is the polarized growth response pathway that has an essential role in remodeling the cell wall at regions of polarized growth during cell proliferation. Consequently, loss of function in this pathway results in cell lysis due to the deficiency in cell wall construction. In this pathway, the yeast protein kinase C isozyme, Pkc1, stimulates the phosphorylation cascade which consists of Bck1 (related to MEKK), the redundant Mkk1 and Mkk2 enzymes (related to MEK) and Mpk1 (related to MAPK).

We exploited these two MAPK activation pathways to examine parameters important for fidelity of signal transmission within a given phosphorylation cascade. We selected for *STE7* and *MKK1* mutations that stimulated their corresponding pathway in the absence of inductive signal. Serine to proline substitutions at analogous positions (Ste7-P^{368} and Mkk1-P^{386}) were recovered, suggesting that this substitution in other MEKs would exhibit similar properties. The Ste7-P^{368} variant has higher basal enzymatic activity than Ste7 but still requires induction to reach full activation. Additionally, Ste7-P^{368} is more permissive in its interactions with MEK-kinases because it gained the ability to transmit the signal from an activated mammalian Raf expressed in yeast. However, Ste7-P^{368} cross function in the growth response pathway occurs only when it is highly overproduced or when Ste5p is missing. This behavior is consistent with recent evidence suggesting that Ste5p is a scaffold for assembly of the mating pathway kinases (3). Our results further suggest that one role for such an assembly is to maintain pathway specificity.

Because polarized growth is also important for projection formation during mating differentiation, we reasoned that activities of the mating and polarized growth response pathways might be coordinated as a part of the mating process. To test this hypothesis we asked if the polarized growth pathway is important for

efficient mating. As predicted, *mpk1* mutant mating mixtures had reduced mating efficiencies compared with wild-type mating mixtures. Pheromone treatment also caused cell lysis in the *mpk1* mutant strain at the time when mating projections were being elaborated. It is noteworthy that the impairments associated with the *mpk1* mutation were observed at the permissive temperature of the strain under proliferative conditions. Finally, we found that pheromone induction causes an 8-fold increase in the activity of Mpk1p. This maximal increase in activity occurs just prior to the time at which projection formation begins. We are now poised to learn how this cross pathway stimulation occurs.

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PATHWAYS INVOLVED IN YEAST CELL POLARITY

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Michael Snyder, Laura Vallier, Jeff Segall, Ellen Flescher, Kevin Madden, Christine Costigan, and Yi-Jun Sheu

Yeast cells undergo polarized growth during both vegetative growth and mating. We have been studying the mechanisms by which yeast cells select sites of polarized growth and execute growth at those sites. The Spa2 protein localizes to sites of cell growth and participates in both bud site selection and projection formation during mating. To attempt to identify other genes which may be important for polarized growth in yeast, genetic screens have been used to identify genes which interact with *spa2*. Five mutants which are synthetically lethal with *spa2* have been identified; these mutations define four complementation groups, *bem2*, *slk1/bck1*, *swi4*, and *cdc10*. We have also identified twelve different genes, which when present in high copy number, suppress the lethality of the *spa2 cdc10* cells. Based on DNA sequence results, these suppressors are likely to correspond to four types of genes: transcription factors, membrane proteins, polarity genes and cell cycle control genes.

Through our analysis of the cell cycle distribution of Spa2p and the phenotypes of *spa2* and *cdc10*, we have suggested models for bud site selection in yeast. In haploid cells, cortical components left from the previous site of cytokinesis may serve to nucleate assembly at an adjacent site on the cortex. Cdc3p, Cdc10p, Cdc11p, and Cdc12p, which have been studied extensively by the Pringle laboratory and may be components of the neck filaments, were found to be important for bud site selection and may be part of the "tag" that marks the site of new cell growth.

We have also been analyzing the mechanisms by which mating yeast cells grow toward their mating partner. When a pipet containing α factor is positioned near a group of wild-type *MATa* cells, the cells arrest division and form projections toward the pheromone source (Segall, PNAS, 1993). We have used this assay to analyze a variety of known mutants for defects in the orientation process. One of the mutants which has been studied extensively is *ste2-T326*, which contains a deletion of most of the sequences in the cytoplasmic tail domain of the *STE2* α-factor receptor gene. *ste2-T326* cells exhibit a delay in projection formation relative to the wild-type cells. Furthermore, although *ste2-T326* cells exhibit some orientation toward the pheromone source, these mutants misorient (i.e. grow away form the pheromone source) at a very high frequency. Preliminary results using a mutant strain with complete deletion of the carboxy terminal domain of Ste2p results in complete loss of orientation. These results indicate that the carboxy terminal tail is important for the directing the site of polarized growth during mating. We suggest that either signals from the receptor and/or the receptor tail itself directly interacts with regulators of the actin cytoskeleton to direct growth toward the "activated" site. In summary, these different data from budding and mating cells are consistent with the view indicate that cortical tags and signals are important for directing polarized growth in yeast.

THE ROLE OF RAS IN THE MATING PROCESS IN FISSION YEAST

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When cells of the fission yeast are starved they leave the mitotic cycle and begin the process of mating. During mating the cells communicate by exchange of mating pheromones that bind to cell surface receptors coupled to a heterotrimeric G protein. Activation of the G protein is thought to stimulate a 'MAP kinase'-like protein kinase cascade consisting of Byr2 kinase (a MAPKKK/MEKK homologue), Byr1 kinase (a MAPKK/MEK homologue) and Spk1 kinase (a MAPK homologue). The fission yeast Ras1 protein, a homologue of mammalian p21ras, is required for the pheromone response and studies from Michael Wigler's laboratory suggest that Ras1 interacts with Byr2.

We have isolated 'activating' mutants of the MAPKK/ MEK homologue Byr1. These mutants bypass the requirement for the MAPKKK/MEKK homologue Byr2 suggesting that they have constitutive activity independent of activation by Byr2 kinase. The activating Byr1 mutants do not, however, bypass the requirement for Ras1 in the mating process even though the pheromone response pathway is fully activated. These experiments show that Ras1 is required to activate two (or more) pathways involved in the mating response. We are now trying to identify components of the other Ras regulated pathway in fission yeast. Preliminary results suggest that there is is an important interaction between this other Ras pathway and the actin cytoskeleton.

II. Signalling Pathways and the Control of Cell Integrity

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Yeast Pkc1p: the protein and its physical interactions

The *S. cerevisiae CLY15* gene, complementing an osmotically remedial cell lysis defect in yeast, was previously shown to be identical to *PKC1*, a gene encoding a yeast homolog of mammalian protein kinase C. Loss of *PKC1* function results in a cell lysis defect that is due to a deficiency in cell wall construction. Addition of osmotic stabilizing agents to the growth medium compensates for the defect and allows cell proliferation.
The Pkc1 protein was shown to associate with the 100,000 x g particulate fraction. This association was resistant to extraction with high salt concentrations, alkali buffer, or nonionic detergents, suggesting that Pkc1p may be associated with a large protein complex. *In vitro*, Pkc1p activity was independent of cofactors such as phospholipids, diacylglycerol, and Ca^{2+}, but mutational inactivation of the pseudosubstrate site of Pkc1p resulted in constitutive activation of the enzyme *in vitro* and *in vivo* [1].

Using a fragment derived from the *S. cerevisiae PKC1* gene as a probe, a homolog of this gene was isolated from the dimorphic pathogenic fungus *Candida albicans*. The predicted *C. albicans* Pkc1 protein shows 51% sequence identity over the entire length with the *S. cerevisiae* Pkc1 protein and was capable of functionally complementing the growth defects of a *S. cerevisiae pkc1Δ* mutant strain on hypoosmotic medium. Deletion of both endogenous copies of the *C. albicans* PKC1 gene resulted in an osmotically remedial cell lysis defect of both the budding and the hyphal growth form and morphologically aberrant cells of the budding form. Despite these abnormalities, the transition between the two growth forms of *C. albicans* occurred normally in *C. albicans pkc1Δ* double disruptants. Similar to its *S. cerevisiae* homolog, *C. albicans* Pkc1p failed to respond *in vitro* to cofactors known to activate several mammalian PKC isozymes.

In order to possibly identify novel components of the *S. cerevisiae PKC1*-mediated pathway, we performed a two-hybrid screen using the *PKC1* gene as the bait-fusion. Among approx. 10^6 yeast transformants, four plasmids were isolated encoding a Gal4(activation domain)::Mkk1 fusion protein. In these proteins, Mkk1p was predicted to start at amino acids 53 or 73, and subsequent experiments showed that full length Mkk1 protein does not interact in the two-hybrid system with Pkc1p, suggesting the existence of a cryptic binding site within Mkk1p. Previous genetic analyses had predicted a third protein, Bck1p, to act downstream of Pkc1p but upstream of Mkk1p [2]. Upon deletion of the *BCK1* gene from the 2-hybrid tester strain, the Pkc1p/Mkk1p interaction was lost but was regained upon retransformation of the strain with a plasmid carrying the *BCK1* gene. These results suggest that the Pkc1, the Bck1 and the Mkk1 proteins form a complex *in vivo*.

1) Watanabe et al., (1994) J. Biol. Chem. 269, 16829-16836
2) Irie et al., (1993) Mol. Cell. Biol. 13, 3076-3083

DISSECTING THE PROTEIN KINASE C / MAP KINASE PATHWAY OF S. CEREVISIAE D. E. Levin, Dept. of Biochemistry, Johns Hopkins Univ., School of Public Health, Baltimore, MD

Mitogen-activated protein kinases (MAP kinases) comprise a family of serine/threonine-specific protein kinases that are activated in response to a variety of extracellular stimuli. The signalling pathways leading to stimulation of vertebrate MAP kinases are activated variously by tyrosine kinases, protein kinase C, RAS, or G proteins. The molecular nature of these pathways is just beginning to emerge. We have identified a novel MAP kinase activation pathway in S. cerevisiae that is mediated by protein kinase C.

The PKC1 gene of S. cerevisiae encodes a homolog of the Ca^{2+}-dependent isozymes of mammalian protein kinase C (1). We have shown that loss of PKC1 function results in a cell lysis defect that is due to a deficiency in cell wall construction (2, 3). Addition of osmotic stabilizing agents to the growth medium compensates for this defect and allows cell proliferation. The PKC1-encoded protein kinase displays a similar substrate specificity to that of mammalian protein kinase C (4). To date, PKC1 is the only protein kinase C-encoding gene known to exist in S. cerevisiae (5).

We have exploited the phenotypic defect associated with loss of Pkc1p function to identify components that function downstream of this protein kinase (6-9). This approach has uncovered several genes that, when mutationally activated or expressed at high levels, suppress the pkc1-associated cell lysis defect. We have proposed that PKC1 regulates a cascade of protein phosphorylation through a bifurcated pathway. On one branch of the pathway are four genes encoding predicted protein kinases that are structurally related to those that function in the mating pheromone-response pathway. BCK1 (Bypass of C-Kinase; 6) is most similar to STE11. MKK1 and MKK2 (MAP Kinase-Kinase; 7) are functionally redundant members of the MAP kinase-kinase (or MEK) family and are also related to STE7. MPK1 (MAP Kinase; 8; Initially designated SLT2) is a member of the MAP kinase family and is also related to FUS3/KSS1. Based both on epistatic relationships among the genes in the PKC1-mediated pathway, and the conserved architecture of the predicted protein kinase cascade with that established for the mating response pathway, we proposed that BCK1 activates MKK1/2, which in turn activates MPK1 (9). Additionally, biochemical evidence supports a model in which PKC1 directly activates BCK1.

The pathway components that function downstream of PKC1 are required for growth at 37°C, but not at 23°C (6-8), therfore, we investigated the role of this pathway in adaptation to heat shock. Wild type yeast cells die rapidly when transfered from 23°C to 50°C. However, a pretreatment at 37°C (for 30 min.) confers resistance to a subsequent heat shock at 50°C. Mutants in MPK1 or BCK1 are no more sensitive to a 50°C heat shock than wild type cells, but are deficient in the adaptation response. Moreover, constitutive activation of BCK1 confers resistance to a 50°C heat shock without pretreatment, suggesting that this signalling pathway is involved in adaptation to elevated temperatures.

In an effort to identify the signals to which the Pkc1p-mediated pathway responds, we have developed an immune-complex protein kinase assay for Mpk1p. Mpk1p is only weakly active in cells grown at 23°C, but is approximately ten-fold more active in cells grown at 30°C, and 100-fold more active in cells grown at 37°C. Moreover, Mpk1p activity is stimulated 170-fold within 30 minutes of heat shock from 23°C to 39°C. This activation is independent of protein synthesis, but is dependent on PKC1 and BCK1 function, confirming our genetic model. Activation of Mpk1p by elevated growth temperature is consistent with the requirement for MPK1 function only at high growth temperature. Other stresses, including low osmotic strength, UV light, hydrogen peroxide, etc., failed to activate Mpk1p appreciably. Interestingly, growth in high osmolarity medium prevented activation of Mpk1p by heat shock.

Many bacterial species respond to elevated growth temperature by altering the composition of their membranes in ways that allow them to maintain constant membrane fluidity at different temperatures. We propose that the PKC1-mediated pathway is required for adaptation to growth at elevated temperatures, and that it may regulate the fluidity of the plasma membrane. We have found that mpk1 mutants are resistant to killing by sodium dodecyl sulfate, consistent with the notion that their membrane composition is different from that of wild type cells. Mutants in this pathway may be unable to maintain a proper interaction between the membrane and the cell wall during growth, resulting in a defect in cell wall construction.

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CHARACTERIZATION OF MAP KINASES INVOLVEMENT IN CELL INTEGRITY OF YEAST SPECIES

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SLT2(MPK1) gene is a MAP kinase homolog of *Saccharomyces cerevisiae*, essential for integrity of cells grown at 37ºC but not at 24ºC (1,2), whose product has been associated with the *PKC1*-controlled phosphorylation pathway on the basis of epistasis relationships (3). The clarification of the function of Slt2p in the signalling pathway is a major interest in this laboratory.

The lack of this gene function determined a sorbitol remediable temperature-sensitive and caffeine-sensitive lytic phenotype, but the genetic background of the strains affected the expression of the lytic phenotype in many respects, such as the extent of growth and lysis at the non-permissive temperature or at different caffeine concentrations. This indicates that the effects of the Pkc1p cascade are directed to the control of the generation of a stable wall structure although the intermediate steps between *SLT2* function and wall functions are unknown. We have followed the release of intracellular proteins, either homologous or foreign, from *slt2* cells at 37ºC and, consistent with the above interpretation, we found that protein release could be prevented in cells growing at 37ºC under osmotic stabilization (with 0.5M sorbitol), whereas the release was instantaneous by eliminating the osmotic protection (4,5).

SLT2 alleles recovered from several *lyt2* strains, originally used to isolate the gene, were shown to be non-functional due to a point mutation determining a change affecting the glycine cluster (Gly-X-Gly-X-X-Gly), which is conserved in protein kinases and other nucleotide binding proteins. By using the "two hybrid expression system" we have also observed that Slt2p interacts *in vivo* with Mkk1p and with Mkk2p, the products of two genes that were suggested to act upstream of *SLT2*, on the basis of epistasis experiments. The N-terminal moiety of Slt2p, containing all the conserved kinase domains, was required both for this interaction and for complementation of phenotypic alterations of *slt2* mutants.

We have isolated a *Candida albicans* functional homolog of *Saccharomyces cerevisiae* gene *SLT2*, that was named *MKC1* (MAP kinase from Candida) and it coded for a putative protein of 58,320 Da with a high degree of identity (55%) with Slt2p(Mpk1p), that displayed all the domains characteristic of MAP kinases. Deletion of the two alleles of *MKC1* gene in this diploid organism gave rise to viable cells that

the opportunistic fungus, that similar to the *S.cerevisiae* Pkc1p cascade, would be involved in cell integrity by controlling the generation of a stable wall structure.

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ROLE OF PROTEIN PHOSPHATASES Z IN THE MAINTENANCE OF CELL INTEGRITY

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Protein phosphatases Z are novel forms of Ser/Thr protein phosphatases and are encoded by two related genes, namely *PPZ1* and *PPZ2*. The complete PPZ1 gene was cloned and characterized in our laboratory [1]. It encodes a large protein (692 residues) whose C-terminal half is about 60% identical to the catalytic subunit of yeast and mammalian type-1 protein phosphatase (PP1c). Its N-terminal half is unrelated to phosphatase sequences and is rich in Ser/Thr, as well as in basic residues. A large number of consensus sites for phosphorylation are also found in this region. The complete ORF of PPZ1 has been expressed in *E. coli* as a soluble GST-fusion protein and found to display activity Ser/Thr phosphatase activity [2]. In some aspects recombinant PPZ1 resembles PP1c, but the enzyme cannot use phosphorylase as substrate. Bacterially expressed PPZ1 can be phosphorylated *in vitro* by different protein kinases at multiple sites, although without significant changes in its activity. Disruption of *PPZ1* results in hypersensitivity to caffeine and this sensitivity is further increased by simultaneous disruption of *PPZ2* (although *ppz2* mutants do not display the phenotype). Exposure to caffeine of *ppz1 ppz2* mutants results in cell lysis that can be prevented by growing the cells in the presence of 1 M sorbitol [3]. PPZ1/PPZ2 phosphatases have been found to be related to the PKC/MAP kinase signalling pathway (i.e. simultaneous disruption of *PPZ1* and *SLT2/MPK1* genes results in non-viable cells) [4]. More recently, we have observed that the disruption of *PPZ1* (but not of *PPZ2*) results in a significant increase in salt tolerance [5]. This effect is not related to alterations in the osmotic mechanisms but to an increased resistance to the toxicity inherent to sodium (and lithium) cations. The possible mechanisms responsible for the observed phenotype will be discussed.

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Osmotic Stress Responses and Signaling Pathways

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Yeast cells are continually exposed to both increases and decreases in osmolarity. Increases in external osmolarity activate a MAP (Mitogen-Activated Protein) kinase cascade called the HOG (High Osmolarity Glycerol response) pathway. This pathway is required for the increased expression of proteins required for osmoregulation and osmotolerance, and also the correct positioning of cell growth and division following an increase in osmolarity. Therefore, HOG pathway mutants grow on low osmolarity medium but not on high osmolarity medium. A second MAP kinase cascade in yeast called the PKC1 pathway is required for cell wall construction. Mutants of this pathway grow on high osmolarity medium but not on low osmolarity medium. The nature of the initial activating signal for this pathway is unknown, although the mutant phenotype suggest the possibility that the PKC1 pathway is activated by low osmolarity. Indeed, the tyrosine phosphorylation of the PKC1 pathway MAP kinase Mpk1(Slt2) increased rapidly in cells following a shift of the external medium to lower osmolarity. The intensity of the response was proportional to the magnitude of the decrease in extracellular osmolarity. This response to hypotonic shock required upstream protein kinases of the PKC1 pathway. Increasing external osmolarity inhibited tyrosine phosphorylation of the PKC1 pathway MAP kinase, a response that was blocked by *BCK1-1*, a constitutively active mutant in an upstream protein kinase. These results indicate that yeast contain two osmosensing signal transduction pathways, the HOG pathway and the PKC1 pathway, that respond to hypertonic and hypotonic shock, respectively.

Yeast signal transduction pathways regulating MAP kinase activation

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Mitogen-activated protein (MAP) kinase kinases (MAPKKs), intermediates in a growth factor-stimulated protein kinase cascade, are dual specificity protein kinases that specifically phosphorylate and activate MAP kinases in response to extracelluar signals. Yeast MAPKK homologs, Mkk1 and Mkk2, participate in a protein kinase cascade activated by Pkc1, protein kinase C homolog. Another MAPKK, Ste7, mediates mating pheromone signal transduction through a pathway activated by βγ subunits of the heterotrimeric G protein. Both pathways contain members of MAP kinase family, Mpk1 and Fus3/Kss1, which are thought to be the immediate downstream target of Mkk1/Mkk2 and Ste7 kinases, respectively, in each signal transduction pathway. We isolated an activated mutation of MKK1, MKK1^{P386}, as a suppressor of pkc1 defect and lies just before kinase domain VIII. Analogous mutation of STE7, STE7^{P368}, also suppressed defect of STE4 encoding Gβ. Without pheromone stimulation, Ste7^{P368} has increased kinase activity and induces some mating responses. Thus, point mutations of yeast MAPKKs cause constitutive activation of the signaling pathways. However, the activity of Ste7^{P368} is still dependent on the presence of the upstream kinase, Ste11. An activated form of mammalian Raf (RafΔN), but not normal c-Raf-1, can substitute for Ste11 activity in vivo. The Raf protein kinase acts as an activator of MAPKK in vertebrates. Raf activation appears to occur downstream of Ras, which is in turn activated by growth factors that signal through receptor protein tyrosine kinases. When c-Raf-1 and Ste7^{P368} are expressed together with mammalian H-Ras, the Ste11 deficiency is rescued by c-Raf-1. Thus, we developed an in vivo system for the assay of Raf activity using the yeast pheromone induced MAPK pathway. These results not only indicate that Raf is

activated by H-Ras in *S. cerevisiae* but also suggest that yeast cells have an activator for mammalian Raf.

To identify yeast components participating in Raf activation, we conducted screens to isolate yeast genes that, when overexpressed, suppressed the transcriptional defect of *ste11Δ STE7^{P368}* cells expressing c-Raf-1. Our expectation was that an excess of a component that functions in Raf activation might enhance Raf activity and, therefore, allow it to substitute for Ste11. We isolated clones from a yeast genomic DNA library on a multicopy plasmid that could activate a mating pathway responsive reporter gene (*FUS1::HIS3*) in a Raf- and Ste7^{P368}-dependent manner. One of them is identical to *BMH1*, which encodes a protein similar to members of the mammalian 14-3-3 family. Bacterially synthesized mammalian 14-3-3 protein stimulated the activity of Raf prepared from yeast cells expressing c-Raf-1. Thus, the 14-3-3 protein may participate in or be required for activation of Raf.

HOG1 dependent and independent processes in yeast osmoadaptation

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The High Osmolarity Glycerol (HOG) response pathway plays an essential role in the adaptation process of yeast cells to hypertonic medium (1). Hog1, a homologue of MAP-kinases, is the ultimate protein kinase in the HOG signalling cascade. No target protein of Hog1 has yet been described at the molecular level. However, it has been shown that the HOG-pathway controls re-establishment of the cytoskeleton after an osmotic shock (2) and the expression of the *GPD1* gene (3). *GPD1* encodes glycerol-3-phosphate dehydrogenase, the key enzyme in glycerol production. Its expression is induced several fold upon osmotic stress leading to enhanced production of glycerol, the compatible solute in yeast. Thus, *GPD1* expression serves as a reporter for HOG-pathway function.

In order to identify downstream components of the HOG-pathway involved in the control of glycerol production or alternative pathways that can take over the function of Hog1, we have isolated suppressor mutations of the osmosensitivity of *hog1Δ* mutants. All mutants characterised so far are recessive and they belong to the same complementation group. In a *hog1Δ* background the mutations confer slow growth on high salt medium, restoration of the cell volume and partial restoration of the morphological alterations under osmotic stress. All mutants produce much higher levels of glycerol than the *hog1Δ* mutant. The mutation is unlinked to *GPD1* and it does not suppress the *gpd1Δ* mutant. In a *HOG1* wild type background the mutations confer slower growth under normal conditions but this effect is independent of glycerol overproduction. This indicates that the mutations affect other processes as well. The mutations do not confer any morphological effects in a *HOG1* strain.

The accumulation of glycerol in the cell depends not only on enhanced glycerol production but also on reduction of glycerol efflux (4). The plasma membrane permeability for glycerol consists of passive diffusion through the membrane bilayer and to about 2/3 of facilitated diffusion through the glycerol facilitator Fps1. The Fps1 channel is inactive under osmotic stress while passive diffusion still occurs. The inactivation of Fps1 is unaffected by deletion of *HOG1* suggesting that this process is independent of the HOG-pathway. Thus, other mechanisms or signalling pathways must exist that are involved in the osmostress response.

A detailed characterisation of the *hog1Δ* suppressor mutations will be presented and the implications for osmoadaptation mechanisms in general and for functioning of the HOG-pathway in particular will be discussed.

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III. Budding and Morphogenic Pathways

Bud-site selection in *Saccharomyces cerevisiae*.

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Cells of the yeast *Saccharomyces cerevisiae* select bud sites in either of two distinct spatial patterns. In the axial pattern, expressed by a and α cells, both mother and daughter cells form new buds near the preceding division site. In the bipolar pattern, expressed by a/α cells, the mother cell can bud either near the preceding division site or near the opposite pole, and the daughter cell generally forms its first bud near the pole distal to the division site. Detailed analysis of budding patterns by fluorescence, time-lapse, and scanning electron microscopy has been used to obtain more precise descriptions of the two budding patterns (Chant & Pringle, 1995). From these descriptions, we conclude that in the axial pattern, the new bud forms directly adjacent to the division site in daughter cells and directly adjacent to the immediately preceding division site (bud site) in mother cells, with little influence from earlier sites. Thus, the division site appears to be marked by a spatial signal(s) that specifies the location of the new bud site and is transient in that it only lasts from one budding event to the next. Consistent with this conclusion, starvation and re-feeding of axially budding cells results in the formation of new buds at non-axial sites. In contrast, in bipolar-budding cells, both poles are specified persistently as potential bud sites, as shown by the observations that a pole remains competent for budding even after several generations of non-use and that the poles continue to be used for budding after starvation and re-feeding. It appears that the specification of the two poles as potential bud sites occurs before a daughter cell forms its first bud, as a daughter can form this bud near either pole. However, there is a bias towards use of the pole distal to the division site. The strength of this bias varies from strain to strain, is affected by growth conditions, and diminishes in successive cell cycles. The first bud that forms near the distal pole appears to form at the very tip of the cell, whereas the first bud that forms near the pole proximal to the original division site (as marked by the birth scar) is generally somewhat offset from the tip and adjacent to (or overlapping) the birth scar. Subsequent buds can form near either pole and appear almost always to be adjacent either to the birth scar or to a previous bud site. These observations suggest that the distal tip of the cell and each division site carry persistent signals that can direct the selection of a bud site in any subsequent cell cycle.

The axial budding pattern appears to be explained in part by the behavior of the *BUD3* gene product. *BUD3* is specifically required for the axial budding pattern: mutations of *BUD3* (including a deletion) disrupt the axial pattern but not the bipolar pattern (Chant & Herskowitz, 1991; Chant *et al.*, 1995). Immunofluorescence localization of Bud3p has revealed that it assembles in an apparent double ring encircling the mother-bud neck shortly after the mitotic spindle forms (Chant *et al.*, 1995). This Bud3p structure persists until cytokinesis, when it splits to yield a single ring of Bud3p marking the division site on each of the two progeny cells. These single rings remain for much of the ensuing unbudded phase and then disassemble. The Bud3p rings are indistinguishable from those of the neck-filament-associated proteins (Cdc3p, Cdc10p, Cdc11p, and Cdc12p), except that the latter proteins assemble prior to bud emergence and remain in place for the duration of the cell cycle. Upon shift of a temperature-sensitive *cdc12* mutant strain to restrictive temperature, localization of both Bud3p and the neck-filament-associated proteins is rapidly lost. In addition, a haploid *cdc11* mutant loses its axial budding pattern upon shift to restrictive temperature. Taken together, the data suggest that Bud3p and the neck filaments are linked in a cycle in which each controls the position of the other's assembly: Bud3p

assembles onto the neck filaments in one cell cycle to mark the site for axial budding (including assembly of the new ring of neck filaments) in the next cell cycle.

The features of the bipolar budding pattern described above suggest that there must be genes whose products are involved specifically in producing this pattern (but not the axial pattern). We attempted to identify such genes by screening for mutants that displayed a specific loss of the bipolar pattern. We mutagenized a bipolar budding *bud3Δ* haploid strain and screened for mutants by Calcofluor staining of individual clones (Zahner *et al.*, 1995). The mutants of interest budded either from random locations (random mutants) or exclusively from one or the other pole (unipolar mutants) in the parental background or in an *a/α BUD3/BUD3* diploid background, but displayed normal axial budding in an *a* or *α BUD3* background. From ~21,000 clones screened, we isolated 11 mutants of interest. The six random mutants proved to contain mutations in six different genes. Two of these genes were *BUD2* and *BUD5*, known from previous work to be necessary for both the axial and bipolar patterns (Chant & Herskowitz, 1991; Chant *et al.*, 1991). Thus, the new mutations appear to affect the ability of Bud2p and Bud5p to interact with bipolar spatial signals but not with the Bud3p-containing axial spatial signal. Two other genes defined by the random mutants are the previously known *SPA2* (Snyder, 1989) and *BNI1* (identified originally on the basis of a synthetic-lethal interaction with *cdc12*: J. Fares & J.R. Pringle, unpublished results); the latter result suggests that the neck filaments may also be involved in the positioning of bipolar spatial signals. The remaining two genes defined by the random mutants are apparently novel (*BUD6* and *BUD7*), and are currently being characterized in molecular terms. The five unipolar mutants included two that bud exclusively from the proximal pole (defining one novel gene, *BUD8*) and three that bud exclusively from the distal pole (defining one novel gene, *BUD9*). The *bud8 bud9* double mutant resembles the *bud8* single mutant, suggesting that the *bud9* phenotype represents a failure to properly position the normal proximal-pole signal rather than an inability to produce this signal. Molecular characterization of both *BUD8* and *BUD9* is in progress.

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GENETIC CONTROL OF CELL POLARITY IN VEGETATIVE AND MATING CELLS OF BUDDING YEAST AND THE FUNGUS USTILAGO MAYDIS.

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We are studying a number of aspects of signal transduction and cell polarity in budding yeast and in *U.maydis*.

Signal transduction in yeast:

PHEROMONE RESPONSE PATHWAY: We have recently found that the MEKK STE11 phosphorylates and activates the MEK STE7 *in vitro* (1). We have now found that STE20, a putative protein kinase in the pheromone response pathway, phosphorylates STE11. The functional significance of this phosphorylation remains to be determined.

PKC1 PATHWAY: We have earlier observed that mutants lacking the SWI4 protein exhibit a growth defect which can be overcome by overexpression of the G1 cyclins, CLN1 and CLN2 (2). We now find that high copy plasmids carrying the *PKC1* gene also allow growth of SWI4-deficient cells. We hypothesize that overproduction of PKC1 stimulates a SWI4-independent pathway of *CLN1* and *CLN2* transcription mediated by the transcription factor MBP1/SWI6. These observations suggest that MBP1 might be direct substrate of the MAP kinase MPK1/SLT2.

Cell polarity:

IN VEGETATIVE CELLS: We have proposed that Bud1p/Rsr1p associates with a member of the Cdc24p group of proteins in a GTP-dependent manner as part of a morphogenetic pathway leading to bud-site selection (3,4). We have observed that Cdc24p associates with Bud1p *in vitro* in a GTP-dependent manner. We have identified additional mutants that exhibit bipolar budding, at least one of which defines a novel gene. Bud4p has been cloned and sequenced and its protein localized using antibodies against Bud4p.

IN MATING CELLS: Mutants of yeast have been identified which exhibit mating defects against certain enfeebled strains (5). These mutants define genes necessary for cell morphogenesis in response to mating pheromone and perhaps for reorientation of cells during mating. The *FAR1* gene, which codes for a cyclin-dependent kinase inhibitor (6), is also necessary for orientation of cells to pheromone.

Signal transduction and cell polarity in *U.maydis*: *U.maydis* is a dimorphic fungus whose haploid form is yeast-like and whose dikaryotic form is filamentous (reviewed in 7). Its a locus codes for pheromones like a-factor of budding yeast and for receptors related to STE3. We have shown that the a locus, and thus the mating pheromones, are necessary for maintenance of the filamentous state; in other words, for an autocrine response.

SIGNAL TRANSDUCTION: A homologue to budding yeast *STE7*, called *fuz7*, has been identified and shown to be required for a-dependent processes such as mating. Strikingly, *fuz7* is also required for induction of tumors in maize, which suggests that it might be a component of a pathway that responds to a plant signal (8).

CELL MORPHOGENESIS: Numerous *ts* mutants have been identified with aberrant morphologies at non-permissive temperature. These may define *U.maydis* analogues and homologues to *CDC24*, *CDC42*, etc.

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MOTOR PROTEINS AND MORPHOGENESIS IN BUDDING YEAST

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Actin (Act1p) and myosins (Myo1p, Myo2p) have been implicated in various aspects of yeast morphogenesis. A temperature-sensitive myosin (*myo2-66*) mutant loses the ability to direct growth to the bud, and instead becomes large and round (Johnston et al., 1991). Govindan et al. (1991) have shown that vesicles build up in the mother cell when the *myo2* mutant is shifted to restrictive temperature, and that this is epistatic to the build-up of secretory vesicles in the bud in late secretory mutants (*sec1*, *sec6*). Thus Myo2p appears to be involved in transport of vesicles to the bud. A puzzling aspect, however, is the failure to detect a concommitant build-up internally of secretory components such as invertase (Liu and Bretscher, 1992; Govindan and Novick, 1993).

A screen for multicopy suppressors revealed that the *myo2* budding defect can be partially corrected by overexpression of Smy1p, a kinesin-related protein (Lillie and Brown, 1992). Furthermore, although deletion of *SMY1* has no detectable phenotype in a wild-type background, the *myo2* mutant requires *SMY1*. It was surprising to find a putative microtubule-based motor substituting or compensating for a defect in an actin-based motor, all the more because microtubules are not normally required for bud growth (Huffaker et al., 1988; Jacobs et al., 1988). However, it is possible that there is a microtubule-mediated pathway that is only required when the major, actin-mediated pathway is defective. We are attempting to explore this possibility by treating the *myo2* mutant with the microtubule-depolymerizing drug, nocodazole. We have also looked for genetic evidence, and have found that mutations in the β-tubulin gene (*tub2*) are not synthetically lethal in combination with *myo2*. Nor do immunolocalization studies (Lillie and Brown, 1994) provide evidence of Smy1p - microtubule interaction; instead there is a correlation with the behavior of actin and Myo2p. Myo2p and Smyp1 colocalize to regions of polarized growth (incipient bud site, tip of small buds, and the mother-bud neck at a late stage of the cell cycle). They may arrive at the bud by traveling along actin cables, which are oriented toward the bud, or they may localize at the bud tip as part of an anchor for the cables, among other possibilities.

Another way to look for an association between Smy1p and microtubules might be to "freeze" Smy1p in place on its substrate. This approach was suggested by studies of Kar3p, another kinesin-related protein in *S. cerevisiae*. Meluh and Rose (1990) found that they could localize a mutant (but not wild-type) Kar3p to microtubules. Since the mutation was in the ATP-binding domain, they postulated that interference with motor activity prevented dissociation of Kar3p from microtubules. Thus, we reasoned that making the same mutation in Smy1p might allow us to localize it to microtubules. We looked carefully, but saw no evidence of such a localization. Instead the mutant Smy1p localized normally. Even more surprisingly, it functioned normally; i.e., plasmid-borne mutant *smy1* suppressed *myo2* temperature sensitivity, and rescued the lethality of *SMY1* deletion in *myo2* mutants. We conclude that either motor activity is not required for Smy1p localization and function, or, contrary to expectation, the mutation has not disabled the motor.

As deletion of *SMY1* does not appear to affect the phenotype, we are screening for mutations that are deleterious in combination with this deletion. The first mutation we identified, using a colony sectoring assay, was in *MYO1*. This was quite surprising, as Myo1p is a very different myosin from Myo2p, and is not expected to be redundant with it. Myo1p is a conventional myosin, with a long α-helical tail that is predicted to dimerize via coiled-coil formation. Conventional myosin dimers aggregate into filaments, which work by sliding actin filaments past one another. Myo2p, on the other hand, may dimerize but is unlikely to form filaments, based on

the behavior of another myosin of its class (Cheney et al., 1993). Furthermore, although both Myo1p and Myo2p may play roles in aspects of morphogenesis, their chief effects appear to happen at different times during the cell cycle: *myo1* mutants have a defect in cell separation so that they grow in clumps and chains (Rodriguez and Paterson, 1990 and our observations), whereas the *myo2* mutant cells can separate but do not bud properly (Johnston et al., 1991). We are investigating why Smy1p has effects on such different myosins.

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A PERMEABILIZED CELL ASSAY FOR THE REGULATION OF POLARIZED ACTIN ASSEMBLY IN BUDDING YEAST. David Drubin, Rong Li, Tom Lila, Shirley Yang and Kathryn Ayscough. Department of Molecular and Cell Biology, University of California, Berkeley, California 94720.

We have established an *in vitro* assay for assembly of the cortical actin cytoskeleton of budding yeast cells. After permeabilization of yeast by a novel procedure designed to maintain the spatial organization of cellular constituents, exogenously added fluorescently-labeled actin monomers assemble into distinct structures in a pattern that is similar to the cortical actin distribution *in vivo*. Actin assembly in the bud of small budded cells requires a nucleation activity provided by protein factors that appear to be distinct from the barbed ends of endogenous actin filaments. This nucleation activity is lost in cells that lack Sla1 and Sla2, two proteins previously implicated in cortical actin cytoskeleton function and demonstrated to be components of the membrane cytoskeleton, suggesting a possible role for these proteins in the nucleation reaction. The rate and the extent of actin assembly in the bud are increased in permeabilized *Δcap2* cells, providing evidence that capping protein regulates the ability of the barbed ends of actin filaments to grow in yeast cells. Actin incorporation in the bud can be stimulated by treating the permeabilized cells with GTP-γS, and, significantly, the stimulatory effect is eliminated by a mutation in *CDC42*, a gene that encodes a Rho-like GTP-binding protein required for bud formation. Furthermore, the lack of actin nucleation activity in the *cdc42* mutant can be complemented *in vitro* by a constitutively active Cdc42 protein. These results suggest that Cdc42 is closely involved in regulating actin assembly during polarized cell growth.

To determine whether Cdc28 plays a direct role in regulating actin nucleation, we tested the effect of Cdc28 activation by the Cln2 protein, one of the G1 cyclins, on cortical actin assembly in permeabilized yeast cells. We found that extracts prepared from G1-arrested cells contain an activity that inhibits the nucleation of cortical actin assembly in the buds of permeabilized yeast cells. This inhibitory activity is eliminated upon activation of Cdc28 by Cln2. Activation of Cdc28 by Clb2, one of the mitotic cyclins, on the other hand, does not have the same effect, consistent with the *in vivo* observation that promoting polarized growth is a function specific for the G1 cyclins.

Control of cell polarity in fission yeast.

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Maintenance of cell shape and polarity is fundamental to proper cell function and differentiation. To identify new genes involved in the control of cell form in fission yeast we have visually screened for temperature sensitive mutants that show defects in cell morphology. 64 mutants have been isolated and linkage analysis has shown that they define 19 independent genes. One group of mutants, called *orb*, show a complete loss of cell polarity and become round when grown at the non-permissive temperature. A second class of mutants shows defects in either selection of the growth site (*tea* mutants) or in the maintenance of growth direction (*ban* mutants). Immunofluorescence analysis of these mutants shows a number of defects in the organization of the microtubule and actin cytoskeleton. In addition we have investigated the function of these genes during the cell cycle and we have shown that although some genes are required to maintain cell shape and polarity throughout the cell cycle, others are required for re-establishment of cell polarity at the conclusion of mitosis.

A *GGP1/GAS1* homolog required for polarized growth in *Candida albicans* is functional in *Saccharomyces cerevisiae*

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The gene *GGP1/GAS1* of *Saccharomyces cerevisiae* encodes a major exocellular glycoprotein of 115 kd (gp115). This protein is synthesized as a 60-kd precursor which is highly modified by N- and O-glycosylation and also by the attachment of a glycosyl-phosphatidylinositol (GPI). The lipid moiety of this structure anchors the protein to the outer layer of the plasma membrane. *GGP1* gene is not essential. However, haploid cells carrying a disrupted *GGP1* gene show several defects. Cells have a lower growth rate, are round, abnormally budded expecially upon entry in stationary phase, resistant to zymolyase, and their viability decreases during growth at neutral pH. These data suggest that gp115 plays a role in morphogenetic events. Recently a homolog of *GGP1* gene, *PHR1*, from the dimorphic pathogen *C.albicans* has been isolated. *PHR1* gene is a *pH-r*esponsive gene that appears to be involved in polarized cell growth in *C.albicans*. The nucleotide sequences of *PHR1* and *GGP1* show a 55% homology whereas the deduced amino acid sequences show a 56% overall identity and 70% similarity. We have analyzed the ability of *PHR1* gene to complement a null mutation in *GGP1* gene. Different constructs were prepared in which the expression of *PHR1* was under the control of its natural or *GGP1* promoter, in the high copy number plasmid YEp24.
Analysis of growth parameters and cell morphologies have indicated a complete complementation of the mutant phenotype. Moreover by Northern blot analysis we have found that when PHR1 gene is under the control of its own promoter is constitutively expressed and is not responsive to the external pH.
Studies are underway to characterize the *PHR1* protein expressed in *S.cerevisiae* and to determine if the signal for GPI attachment is functional in *S.cerevisiae*..

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IV. More Morphogenesis and Signalling

DIMORPHISM IN YEAST: A MODEL FOR FUNGAL DEVELOPMENT

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Saccharomyces is dimorphic, capable of growth both as yeast-form (YF) and filamentous pseudohyphal (PH) cells. We used video microscopy to compare YF and PH cells grown under nitrogen limitation. The generation times of YF and PH were nearly equal but their growth patterns were distinct. YF cells divide by bipolar budding and display an asymmetric division pattern. Mothers always budded before daughters, performing a Start controlled cell cycle. Budding of PH cells is unipolar and division is symmetric. Subsequent budding of mother and daughter is synchronous. The PH budding pattern was dependent upon an intact MAP kinase signaling cascade—*ste7* and *ste20* mutants displayed asymmetric, asynchronous budding. Unlike YF growth, in PH cells the budded period covers nearly the entire cell cycle. Linear growth of PH cells is restricted to the budded period. Thus, Start control of cell cycle progression at G1/S is bypassed. Immunofluorescence of DNA and tubulin distribution suggest that M phase is delayed until cell size is doubled. Flow cytometry and cell sorting show that most budded PH cells have fully replicated DNA, supporting a G2/M restriction point. PH maintain their division pattern independent of growth rate, ruling out a temporal delay in G2. PH cell size is regulated chiefly at the G2/M transition. Mutation of the *CDC28* phosphorylation site Y19 does not perturb PH growth. PH cell-size control may depend upon novel effectors of the *STE* pathway inhibiting cell cycle progression at G2/M.

G1 Cyclins are Stabilized in *grr1* Mutants and During Pseudohyphal Growth of *Saccharomyces cerevisiae*.

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We're interested in studying the functional significance and the biochemical mechanism of G1 cyclin proteolysis. We found that instability was conferred on β-galactosidase in yeast by fusion to the CLN1 G1 cyclin. The fusion protein was stabilized by deletion of PEST-rich sequences of CLN1 that are necessary for its instability. We isolated mutants in which the CLN1-β-galactosidase fusion protein was stabilized using a colony color assay to monitor β-galactosidase activity. Endogenous CLN1 and CLN2, but not CLN3, were stabilized in a complementation group of mutants that proved to be alleles of the previously characterized *GRR1* gene. *grr1* mutants are defective in glucose repression, glucose and divalent cation transport and exhibit a pseudohyphal-like growth. A suppressor analysis indicated that G1-cyclin stabilization is probably not an indirect consequence of the nutrient uptake defects of *grr1*. We propose that GRR1 is a component of a regulatory pathway linking nutrient transport and G1 cyclin turnover.

The *grr1* phenotypes suggested to us that G1 cyclin stability might be modulated during pseudohyphal differentiation of wild-type yeast cells. We found that CLN1 was partially stabilized when wild-type cells underwent pseudohyphal differentiation in nitrogen-limited liquid cultures. Furthermore, deletion of *CLN1* inhibits pseudohyphal differentiation whereas the overexpression of *CLN1* sufficed to induce pseudohyphal-like growth in wild-type diploid cells growing in a rich nutrient medium. Thus, the degradation of the G1 cyclins is regulated such that their instability couples cell growth to cell division in the unicellular state whereas their stabilization is implicated in pseudohyphal differentiation.

Involvement of the Ras/cAMP signal transduction pathway in the maintenance of cellular integrity in yeast.

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All unicellular organisms which have rigid cell walls face the problem of increasing the size of that wall by the insertion of new material while maintaining their osmotic integrity. This process of wall extension must be carefully integrated with the overall control of cell growth and division (22). In this report, we will present data which indicates that the Ras/cAMP signal transduction pathway is involved in this integration process and plays a major role in the maintenance of the structural integrity of cells of the yeast,*Saccharomyces cerevisiae*.

cAMP is a key "second messenger" which plays an important role in the regulation of a variety of cellular processes. In the lower eukaryote, *Saccharomyces cerevisiae*, only one biochemical function has, so far, been ascribed to cAMP: the activation of cAMP-dependent protein kinase A. This enzyme controls the phosphorylation of a number of different target proteins and, in this way, modulates their activity and function. cAMP is produced by adenylate cyclase which, in *Saccharomyces cerevisiae* , is a peripheral membrane protein (11) encoded by the gene *CDC35/CYR1* (3, 10). The activity of adenylate cyclase is, in turn, regulated by the the Ras1 and Ras2 proteins (4, 14).

The Ras-adenylate cyclase relationship in yeast has been the subject of intensive study which has resulted in the definition of one of the organism's signal transduction pathways. This Ras/cAMP - dependent signal transduction pathway has a number of other components in addition to the *RAS1, RAS2* and *CYR1/CDC35* genes. The cellular concentration of cAMP is determined by the balance between the nucleotide's synthesis by adenylate cyclase and its degradation by two phosphodiesterases, specified by the *PDE1* and *PDE2/SRA5* genes (12, 16). The activity of Ras is modulated by *CDC25* (1, 2), a gene whose product activates Ras by promoting the removal of bound GDP and its replacement with GTP, and by *IRA1, IRA2* and *MSI1/JUN1*, all of which appear to down-modulate Ras activity by stimulating that protein's intrinsic GTPase activity (15, 18, 19). The interaction between the Ras proteins and adenylate cyclase is promoted by the*SRV2/CAP* gene (5, 6) whose gene product also mediates an interaction with the cytoskeleton (8, 24). Finally, the genes encoding the catalytic (*TPK1, TPK2* and *TPK3*) and the regulatory (*BCY1*) subunits of the cAMP-dependent protein kinase have been defined (20, 21).

The effects of mutations in the above-mentioned genes on the physiology of the yeast cell have been studied in detail. Most of these mutations result in distinct and severe phenotypes, for example: loss of viability (*ras1 ras2* double mutants); heat shock and starvation sensitivity (*bcy1*); temperature sensitivity (*cdc25; cyr1*); failure to utilise non-fermentable carbon sources (*tpk2 tpk3 bcy1* or *tpk1 tpk3 bcy1* triple mutants); inability to grow on the rich medium YPD (*srv2/cap*). In contrast, defects in either *PDE1* or *PDE2* (the genes encoding the low and high affinity cAMP phosphodiesterases, respectively) have not been reported to result in any dramatic phenotypic changes. They cause only very modest increases in the level of intracellular cAMP (12, 16) and have a nearly wild-type phenotype under most conditions. Nevertheless, mutations in *PDE2* (*pde2::URA3; rca1*) were shown to rescue otherwise non-viable *ras1 ras2* double mutants on rich medium in the presence of exogenous cAMP (25, 26). A recent report (7) suggested that cAMP regulates the ability of the polar-budding, diploid yeast cell to undergo a morphological transition to the pseudomycelial, "foraging", growth habit. This morphological change occurs upon nitrogen limitation and is potentiated by mutations, such as $RAS2^{Val19}$, which result in elevated steady-state levels of cAMP. These data implicate the Ras/cAMP-dependent signal transduction pathway in the control of the pattern of cell surface development. We will provide evidence that this signal transduction pathway also has an influence on an even more fundamental role of the yeast cell surface: the maintenance of the structural integrity of the cell itself.

We will show that the *PDE2* gene of *Saccharomyces cerevisiae*, which encodes the high affinity cAMP phosphodiesterase, is an extragenic suppressor of the osmotic fragility mutation, *srb1-1* (23). Disruption of *PDE2*, but not *PDE1* (the gene for the low affinity enzyme), simultaneously confers the following phenotypes on yeast cells: susceptibility to lysis upon osmotic shock, increased sensitivity to antibiotics, and competence of intact cells for DNA-mediated transformation without lithium treatment. Mutation in another constituent gene of the Ras2/cAMP dependent signal transduction pathway,*RAS2 Val19*, is found to result in the same set of phenotypes which are also shared by *srb1-1* mutants. The latter, in addition, are unable to grow in the absence of an osmotic stabiliser (13, 17, 23). These mutations, including *srb1-1*, all result in elevated cAMP levels and the severity of the different phenotypes shows a reasonable correlation with the concentration of cellular cAMP. However, the failure of *pde1* mutants to display the phenotypes, as well as the interaction of these different genes in double mutant strains, suggests that gross cAMP concentrations are not the sole determinant of these effects; the mode or site of production of the nucleotide may be important. These unresolved questions probably require the characterisation of the *SRB1* gene itself for their resolution. We have recently isolated a clone from a single copy yeast genomic library which complements the *srb1-1* mutation. The characterisation of this clone will be described.

The osmotic fragility and increased permeability of *srb1-1* ,*RAS2 Val19* and *pde2* mutants also has practical utility. In particular, we (9) have demonstrated that intact *pde2* mutant cells may be transformed with high molecular weight YAC DNA without recourse to the use of lithium ions. This no-lithium transformation technique is less recombinagenic, and shows a lower co-transformation frequency, than the traditional spheroplast transformation method. These two features should reduce the incidence of chimera formation when constructing YAC libraries of large genomes and, moreover, considerably facilitate the automation of post-transformation steps in the construction of such libraries. These, and other, applications of the mutants will be discussed in the presentation.

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Three *Saccharomyces cerevisiae* mutants showing autolytic phenotype and morphological defects.

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In order to study the genes implicated in the progression in the mitotic cycle, morphogenetic events and cell wall integrity, several *S. cerevisiae* temperature sensitive mutants were isolated.

The selection criteria was the ability to lyse and release their intracellular contents at the restrictive temperature of 37°C . We will focus the attention in this talk in three of these mutants; one was generated from the haploid strain S288C (lyt 1) and two from the diploid strain D1 (327 and 918).

Mutation *lyt1-1* is pleiotropic: 1-cells lyse when the bud has the size of the mother cell . 2- Haploid strains have, at 37°C, the budding pattern of diploid strains. 3- Homozygous diploid strains are unable to sporulate, even at the permissive temperature.

A clone able to complement all these phenotypical traits was isolated after transformation with a genomic library bound to a centromeric vector; and carried genes *CDC15* and *NPK 1*. Gene *CDC 15* complements autolytic and CDC phenotypes, but needs the presence of gene NPK 1 to accomplish sporulation.

Three suppressor genes were also cloned using a *S. cerevisiae* genomic library, and two human and fission yeast cDNA libraries bound to episomic vectrors. *SPO 12* was the gene isolated from *S. cerevisiae* and it is able to complement *lyt 1* mitotic phenotypical traits. The other two clones were only able to complement the defect in sporulation.

In the mutants generated from D1, lytic phenotype was accompanied by morphogenetic defects. Mutant 327 has unusually long buds, a failure in cytokinesis, and an increase in the sensitiveness to nikkomicin. *CDC10* gene complemented all the phenotypical traits of this pleiotropic mutant. The *CDC10* allele present in 327 has been rescued, and chimaeric genes are being constructed to map the putative mutation/s.

Mutant 918 cells are quite heterogeneous in size, some of them very large, filled with vacuoles, show a random budding pattern and often are multinucleated. Cell wall integrity is impaired in strains carrying this mutation, and multinucleated diploid 918 cells sporulate in the presence of 1M-sorbitol leading to asci containing multiple spores. A 9kb DNA fragment which complements both phenotypical traits (cell size and polispores formation) was isolated from a *S. cerevisiae* genomic library in a centromeric vector .

The Ca^{2+}/Calmodulin-Dependent Protein Phosphatase Calcineurin Acts in a Signaling Pathway Required for Ion Homeostasis.

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We use *Saccharomyces cerevisiae* as a model to identify genes required for adaptation to high salinity. Yeast mutants highly sensitive to NaCl are being used to isolate the wild-type genes both from yeast and plants by gene complementation. One of these genes encodes the regulatory subunit of the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin. We have shown (Mendoza *et al.*, 1994) that calcineurin activity is required for the transition of the K^+ transport system from the low-affinity to the high-affinity state. The high-affinity state of K^+ transport develops in wild-type cells in response to Na^+ stress, thereby allowing a better discrimination between Na^+ and K^+ ions, and by K^+ starvation. Calcineurin deficient mutants switch normally to the high-affinity state upon K^+ deprivation but not when the cells are stressed with Na^+. These results implicate two convergent signaling pathways leading to the activation of the high-affinity K^+ transport state, only one of them being calcineurin-dependent. Calcineurin is also needed for the expression of the *ENA1* gene that encodes a P-type ATPase involved in Na^+ efflux that is induced by NaCl stress in wild-type cells. Calcineurin mutants show a reduced accumulation of *ENA1* mRNA due to an impaired expression of the *ENA1* gene promoter. Therefore, calcineurin seems to play a key role in a Ca^{2+}-dependent signal transduction pathway leading to a coordinated response of influx and efflux transporters for appropriate ion homeostasis under NaCl stress. This Ca^{2+}/Calcineurin-based pathway would be complementary to the MAP kinases cascade invoked by increased external osmolarity and high salt (Brewster *et al.*, 1993; Maeda *et al.*, 1994) because calcineurin mutants are specifically sensitive to Na^+ ions but are not osmosensitive.

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V. Cell Wall Biosynthesis and Dynamics

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MORPHOGENESIS OF THE FUNGAL CELL WALLS: THE PIVOTAL ROLE OF CELL WALL PROTEINS.

The fungal cell walls are tough, stable but also dynamic structures as change continuously through the cell cycle and in response to external stimuli. In addition some fungal species adopt alternative morphology in response to changes in external environment.

Two important questions may address us: What are the advantages that lead the microorganisms to change their cellular morphology?, What are the molecular mechanisms involved in cellular morphogenesis?.

The steps involved in the regulation of the cellular morphology may be the following: 1. Changes in "morphogens" present in the growth environment. 2. Reception and transduction of the corresponding signals. 3. Differential expression of genes, 4. Synthesis and secretion of "morphogenetic" proteins and formation of microfibrillar polymers, and 5. Interaction and assembly of macromolecules to produce the final architecture of the cell walls.

The present talk will be focused on the last point but basic information about cell wall organization will be also discussed. ß-glucans are the main structural polysaccharides of the cell walls where chitin is a minor component. The proteins of the fungal cell walls may be divided in two groups, one is formed by those loosely associated whereas the other by proteins covalently linked to the structural polysaccharides (chitin and ß-glucans).

Hydrolases and chemical reagents have allowed the controlled degradation of isolated cell walls and poly- and monoclonal antibodies have help to dissect the processes of incorporation of specific molecules in the cell wall.

The results obtained have shown that all cell wall macromolecules are highly interconnected.

The presence of specific proteins in the cell walls in parallel with changes in cellular morphology suggests that they could determine how other polymers interact and assemble. As a consequence, the wall proteins may be responsible of the molecular organization of the cell walls and of the fungal cell morphology.

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Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37 degrees C.
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REGULATION OF CELL WALL ß 1, 6-GLUCAN IN *Saccharomyces cerevisiae*. Howard Bussey, Jeffrey L.Brown, Bo Jiang and Terry Roemer, Department of Biology, McGill University, Montreal, Canada.

We have explored synthesis of ß 1, 6-glucan, to attempt to understand how cell wall polymer synthesis is integrated with growth and morphogenesis. This essential polymer is made sequentially in the yeast secretory pathway, and is a glucosyl moeity of glycoproteins. Genes whose products participate in the synthesis of this glucan are found cytoplasmically, in the endoplasmic reticulum, in the Golgi, and at the cell surface. Recent results indicate that inputs from multiple signal transduction pathways regulate ß 1, 6-glucan assembly. Kre6p is an integral-membrane Golgi phosphoglycoprotein necessary for ß 1, 6-glucan synthesis, probably as a glucan synthase component. Kre6p when overproduced can partially suppress the osmotic dependence of a *pkc1* null mutation, and when deleted with *pkc1* causes synthetic lethality. *pkc1* mutants have reduced levels of cell wall polymers and, taken with these suppression and synthetic lethal interactions, our results suggest that *PKC1* participates in cell wall assembly by regulating the synthesis of cell wall components including ß 1, 6-glucan.

EXG1 encodes an cell wall glucanase/transglycosylase implicated in maintaining ß 1, 6-glucan integrity. Transcription of *EXG1* is modulated by the *PBS2* product, a Map kinase kinase associated with the HOG osmosensing pathway. Epistasis experiments indicate that *PBS2* regulation of *EXG1* expression is negatively regulated by the *PTC1* product, a type 2C protein phosphatase, and occurs in a *HOG1*-dependent manner.

Studies on suppressors of mutations in *KRE9*, a gene encoding a cell wall protein involved in ß 1, 6-glucan synthesis, have identified *SKN7*, a gene encoding a functional bacterial two component system implicated in the regulation of the yeast extracellular matrix. Skn7p contains a potential "receiver motif" homologous to that found in bacterial response regulators, signal transducing effector proteins regulated by phosphorylation at conserved aspartate residue coresponding to D427 in Skn7p. Mutations at D427 can lead to diminished or enhanced activity of Skn7p function as a transcription factor, providing evidence that a receiver motif functions in regulating the activity of an effector protein in a eukaryote. Skn7p, when overproduced, also suppressed growth defects associated with a *pkc1* mutation. However, epistasis experiments indicate that Skn7p does not appear to function directly downstream of the *PKC1*-MAP kinase pathway. Rather, Skn7p may function in a two component signal transduction pathway that acts in parallel with the *PKC1* cascade to regulate growth at the cell surface.

BIOSYNTHESIS OF CHITIN AND ITS REGULATION IN *S. cerevisiae*. *CAL3*, A GENE INVOLVED IN CHITIN SYNTHESIS, IS ALSO REQUIRED FOR MATING.

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Three different enzymatic activities have been implicated in cell wall chitin synthesis in *S. cerevisiae*, chitin synthase I, II and III. There is some information about their function along the life cycle of *S. cerevisiae* but not much is known about the mechanisms by which these activities are regulated.

Chitin synthase III activity is responsible for the majority of the synthesis of cell wall chitin in *S. cerevisiae*. Three different genes have been detected, so far, to be required for chitin synthase III activity *in vitro*: *CAL1≡CSD2≡DIT101≡KTI2*, *CAL2≡CSD4* and *CAL3* (*CAL* term, for this genotype, has been withdrawn and a new generally accepted nomenclature has been adopted for these genes i.e.: *CHS3*, *CHS4* and *CHS5* respectively, standing for *Ch*itin *s*ynthesis).

CHS3 is most probably a structural component of the chitin synthase III activity and no precise function has been reported, up to now, for *CHS4* and *CHS5* genes. In this presentation we report on the isolation and characterization of *CHS5* (previously, *CAL3*). It is confined in an open reading frame of 2013 nucleotides, encoding for a protein of 671 amino acids long with an estimated molecular mass of 73642 dalton, and located on the right arm of chromosome XII close to *cdc3* locus. The predicted amino acid sequence shows two peculiar features: a string of 10 heptameric repeated blocks (472 to 541 residues) and a carboxy-terminal tail of several lysine residues. *CHS5* is responsible for complementing all the phenotypes ascribed to *cal3≡chs5* mutant i.e.: resistance to calcofluor and defect of chitin synthesis both *in vivo* and *in vitro*. Overexpression of *CHS5* does not increase chitin synthase III activity level in vitro. Northern analysis indicates the presence of an mRNA of 2.1 Kb size whose expression pattern appears to be constitutive. Deletion of *CHS5* is not lethal but, as compared to isogenic strains, promotes a severe defect in mating in homozygosis. Electron microscopy observations and cytoduction experiments lead to the conclusion that *CHS5* is involved in a very early stage of mating, besides its role in chitin synthesis.

CELL CYCLE-DEPENDENT REGULATION OF β-(1->3)-GLUCAN SYNTHESIS IN *SACCHAROMYCES CEREVISIAE*

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In a search for cell wall mutants, two Calcofluor White-hypersensitive mutants (*cwh52* and *cwh53*) were identified which showed a reduction in the amount of cell wall glucose of 50 and 75%, respectively, indicating that the synthesis of β-(1->3)-glucan was severely affected (1). This was supported by the observation that both mutants had become resistant to K9 killer toxin. In contrast, both mutants remained sensitive to K1 killer toxin indicating that their β-(1->6)-glucan levels were not significantly affected. The corresponding genes were isolated by screening for wild-type resistance to Calcofluor White. Partial sequence analysis of the plasmid able to complement the *cwh52* mutation showed that it contained the *GAS1/GPP1* gene. This gene encodes a 125-kd protein with an unknown function that is anchored to the plasma membrane by a GPI anchor (2, 3). Cloning and sequencing of the plasmid complementing the *cwh53* mutant showed that it contained a new ORF of 5628 base pairs, encoding a protein of 1876 amino acids with a molecular mass of 215 kd. A data base search revealed no homology to any known sequence. Its sequence predicts that it is an integral transmembrane protein with approximately sixteen membrane-spanning domains organized into two groups of transmembrane domains separated by a large hydrophilic loop.

Northern analysis showed that the transcript levels of *CWH53* varied strongly during the cell cycle and rapidly increased in late G1. Interestingly, the *GAS1/GPP1* mRNA is also highly regulated during the cell cycle peaking late in G1 before induction of H2A mRNA and bud emergence (4). These observations suggest strongly that the synthesis of β-(1->3)-glucan is strictly cell cycle-regulated. Additional evidence was obtained by studying the terminal phenotypes of cells growing in the presence of lethal concentrations of Calcofluor. Whereas wild-type cells stopped growing in all phases of the cell cycle, some mutant alleles of both *cwh52* and *cwh 53* were arrested just after bud emergence indicating a specific role for both genes in β-(1->3)-glucan synthesis during initial bud growth. Since *pkc1* cells show a similar cell cycle arrest (5) and are also affected in β-(1->3)-glucan synthesis (6), both Gas1p and Cwh53p might be downstream targets of the *PKC1* signal transduction cascade. This is under investigation.

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O-Glycosylation in Saccharomyces cerevisiae

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Pmt1p (protein mannosyltransferase 1) - an integral endoplasmic membrane protein - catalyzes the initial reaction of protein O-glycosylation in *Saccharomyces cerevisiae*. This protein was purified to homogeneity. The 92 kDa N-glycosylated protein transfers mannose residues from dolichyl phosphate-D-mannose to specific serine/threonine residues of proteins, a type of mannosyltransfer only observed in fungal cells so far. The corresponding gene has an open reading frame of 2451 bp. The predicted protein consists of 817 amino acids including three potential N-glycosylation sites. The hydropathy blot indicates a tripartite structure of the protein: an amino-terminal third and a carboxyl-terminal third, both with multiple potential transmembrane helices and a central hydrophilic part. Gene disruption led to a complete loss of in vitro mannosyltransferase activity. In vivo, however, protein O-glycosylation decreased only to about 40%-50% (Strahl- Bolsinger et al. 1993), which indicates at least one additional mannosyltransferase. Recently another gene (*FUN 25*) was found by sequencing a part of chromosome I with a sequence homology of about 30 % in comparison to *PMT1* and a similar hydropathy blot (Lussier et al. 1994).

PCR experiments with primers from homologous regions of *PMT1* from *Saccharomyces cerevisiae*, *FUN 25* and *PMT1* from *Kluyveromyces lactis* (Fleer, unpublished) yielded two new genes being homologous to *PMT1* and *FUN 25*. We are about to sequence and disrupt the new genes. Characterization of the received mutants will show, whether these gene products play a role in O-glycosylation. Simultaneously gene disruption of all mannosyltransferases participating in the transfer of the first mannose to O-glycosylated proteins will answer the question whether O-glycosylation is an essentiell process in yeast.

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POSTERS

INVOLVEMENT OF Slt2p (Mpk1p) IN THE MAINTENANCE OF A STABLE CELL WALL IN *S. cerevisiae*.

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Morphogenesis of yeast cell depends on the synthesis, assembly and controlled autolysis of cell wall components to generate and maintain an stable structure which is responsible for cell integrity. The use of a *S. cerevisiae* autolytic mutant led us to clone a protein kinase gene (*SLT2*) by complementation of its thermosensitive and sorbitol-rescued lytic phenotype, as a first evidence of its involvement in the maintenace of an osmotically stable cell wall. This gene (also named *MPK1)* codes for a MAP kinase homolog which has been implicated in a PKC-mediated signal transduction pathway. The members of the MAP kinase family play an important role in the control of cellular functions in diverse organisms, as the last component of an evolutionary conserved cascade of protein kinases.

By phenotypic characterization of null mutants and mutants modified in functional aminoacid residues of Slt2p we have demonstrated that cells have a fragile cell wall when exposed to high temperature or low caffeine concentrations, so they can only survive in an osmotically stabilized medium, and a change to a hypotonic medium led to a rapid (instantaneous) cell lysis with the release of the intracellular content. This observation and the ability of a double mutant *slt2 hog1* to grow at high temperature in the presence of sorbitol indicates a direct protection of the osmotic stabilizer instead a complementation by another MAP kinase homolog (Hog1) which is induced by hypertonic stress. We have also shown that *slt2* mutants displayed higher sensitivity than isogenic strains to several inhibitors of cell wall synthesis such as cilofungin, nikkomycin and calcofluor, as expected in cells with a weaker cell wall.

The phenotypic features of these mutants allowed us to explore a potential biotechnological application by developing a system based on the release of the bulk of intracellular proteins leaving behind cell ghosts and debris. This system could be useful for obtaining large-scale heterologous protein preparations reducing downstream processing.

DUAL REGULATION OF TREHALASE ACTIVITY BY THE RAS-cAMP PATHWAY AND GLUCOSE REPRESSION IN Saccharomyces cerevisiae

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Over the past years, the study of several yeasts has been chosen as an alternative model for getting more insight on the role of RAS genes in mammals. *Saccharomyces cerevisiae* contains two RAS genes highly homologous to the human ras genes, which are involved in oncogenic transformation. Exclusively in this yeast, RAS proteins appear to control adenylate cyclase. On the contrary, in humans RAS modulate a mitogen activated protein kinase cascade (MAP), which plays a crucial role in the control of cellular growth .

In *S. cerevisiae*, the RAS-adenylate cyclase pathway acts as a nutritional transmission signal. Resting cells sense the presence of extracellular fermentable sugars by increasing their intracellular content of cAMP. In turn, this cAMP peak promotes a cascade of reversible protein phosphorylation carried out by cAMP-dependent protein kinases (cAPKs). The so-called neutral trehalase (NTH1) is one of the key enzymes phosphorylated and subsequently activated by cAPKs. The active trehalase is responsible for the enzymatic breakdown of stored trehalose that takes place under different physiological and metabolic situations.

On the other hand, *S. cerevisiae* contains another trehalase located into the vacuoles. This enzyme was only detectable in glucose-grown resting cells or in cultures growing on respiratory carbon sources (glycerol or ethanol). The presence of cycloheximide completely blocked the appearence of the enzyme. The vacuolar trehalase was partially derepressed in the mutant hex2, which is deficient in glucose repression. Addition of fresh YPD medium to stationary-phase cultures provoked the slow and irreversible inactivation of vacuolar trehalase. Hence, glucose repression is proposed as the regulatory mechanism for this second type of trehalase.

STUDY OF AUTOLYTIC MUTANTS 327 AND 918: IMPLICATIONS IN CELL INTEGRITY AND MORPHOGENETIC CONTROL

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In order to uncover new fuctions related to cell wall integrity we isolated from a diploid *S. cerevisiae* strain several temperature sensitive mutants able to lyse and release their intracellular content at the restrictive temperature of 37°C (1). Further studies were performed in two of these mutants, 327 and 918, whose lytic phenotype was accompanied by morphogenetic defects.

At a non-permissive temperature, mutant 327 showed unusually long buds, a failure in cytokinesis, and an increase in the sensitiveness to nikkomicin. We found that *CDC10* gene complemented all the phenotypic traits of this pleiotropic mutant. This gene encodes one of the putative components of the 10nm microfilaments ring, which stands in the internal part of the membrane in the region below the chitin-rich ring at the mother-daughter junction (2,3,4). Northern Blot analyses reveal that CDC10 mRNA is expressed in the mutant. The CDC10 allele present in 327 has been rescued, and chimaeric genes are being constructed to map the putative mutation/s in the coding region of 327's CDC10 gene. In order to identify other genes involved in the regulation or function of CDC10, we are also carring out the transformation of this mutant with cDNA libraries included in vectors that promote overexpression .

Unlike 327, 918 mutant was able to sporulate so segregational studies were performed, leading to the conclusion that it carried a recessive mutation responsible for a peculiar phenotype: cells are quite heterogeneous in size, some of them very large, filled with vacuoles, showing a random budding pattern and often multinucleated. Spores carrying this mutation were remarkably sensitive to several antifungal drugs, among them echinocandin B and papulacandin B, which affect glucan synthesis. Phenotypic characterizations show that cell wall integrity is impaired in strains bearing this mutation. Multinucleated diploid 918 cells sporulate in the presence of 1M-sorbitol leading to asci containing multiple spores, which we call "poliads".

We have isolated a 9kb DNA fragment from a *S. cerevisiae* genomic library in a centromeric vector which complements both phenotypical traits: cell size and poliad formation.

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Sur1, suppressor of _rvs_ mutants, also suppress the thermosensitivity of _act1-1_ and synthetic lethality of _rvs161Δ_, _act1-1_ mutants. **Desfarges L., A. Breton, M. Bonneu, M. Crouzet, P. Durrens, J.C. Le Ball, P. Navarro, P. Sivadon and M. Aigle. Laboratoire de génétique, UPR CNRS 9026, Allée des facultés, F33405 TALENCE.**

Mutations in *RVS161* and *RVS167* genes have been selected on the basis of an inability to stop budding upon starvation [1]. These mutations also confer a loss of cell viability together with an abnormal and heterogeneous cell morphology upon starvation conditions. The *rvs* mutants exhibit the same pleiotropic phenotypes as actin deficient strains. In particular, these mutants display abnormal distribution of actin, random budding in diploid cells and the inability to carry on endocytosis (H. Riezman, personal communication).

In N-terminal region of the two Rvsp, the proteins sequences share similarity and define the RVS domain which is found in chicken amphiphysin [2], a synaptic vesicle associated protein with SH3 domain. Moreover, similarities are found between the Rvsp and human myosin and tropomyosin protein sequences. Rvs167p contains an SH3 domain in the C-terminal region, which is found, in particular, in several proteins that interact with actin such as Bem1p, Abp1p and Sla1p [3]. Besido, using the two-hybrid system to identify yeast protein that can bind with actin, Rvs167p was found along with actin, profilin and Srv2p [4]. These recent data suggest Rvsp could physically bind actin. Finally, some diploids (*rvs161/+*, *act1/+*) reveal non allelic non complementation which is dependent on actin gene allele. The (*rvs161*, *act1-1*) double mutant displays synthetic sub-lethality. These common defects shared by *rvs* and *act1* mutants, the non allelic non complementation and the selection of Rvs167p by two-hybrid system described above, indicate that Rvsp and actin are implicated in a common function.

To learn more about *rvs* function, we selected physiological suppressors of *rvs161Δ* mutation [5]. Four complementation groups named *SUR1, 2, 3* and *4* were defined. In each case, the *sur* mutations suppress the (*rvs161*), (*rvs167*) and (*rvs161*, *rvs167*) defects, hence theses genes are implicated in the same biological function. The *sur1* mutation suppresses the random budding defect in *rvs161* diploid, the endocytosis defect and the abnormal distribution of actin in an *rvs161* genetic background. The *sur* mutations also show abnormal morphologies in early stationary phase (e.g. elongated buds).

In *act1-1* background the *sur1* mutation suppresses different effects : i) the sensitivity to salt of *act1-1*, ii) the thermosensitivity at 37°C of *act1-1*, iii) the sub-lethality of (*rvs161*, *act1-1*) mutant.

The *sur1* gene is not an essential gene and its deletion is a suppressor allele as the initial mutation. Sur1p is a putative protein of 382 amino-acids with a pHi of 9.3. The low transcription and translation rates agree with the low codon bias index (0.08). Furthermore, a Mata1p/Matα2p repressor binding site consensus sequence was found in the *SUR1* promoter. This gene could be regulated by mating type although no regulation was found in the tested conditions, so far.

In summary, the *rvs* mutants share numerous defects with *act1* mutant and could be implicated in a function of stabilization of actin cytoskeleton. Because the *sur1* mutation alleviates *act1-1* defects, suppresses the abnormal distribution of actin in *rvs* mutant and in particular conditions leads to elongated shape cell, this suggests that Sur1p could be a regulator of stabilization or reorganization of actin cytoskeleton.

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ABSTRACT

Protein tyrosine phosphorylation and glycosylation in *C. albicans*. Role of GRB2- and SOS-like proteins in cell growth and myceliation

We have studied growth and germ tube formation in *C. albicans* under several cell culture conditions. It was found that:

1- In the presence of a carbon source, Ca^{2+} and Mg^{2+} stimulate cell growth at 28°C in a pH-dependent manner.

2- At 37°C, *C. albicans* can generate germ tubes in a cation-free medium, instead, addition of Mg^{2+} accelerates, but Ca^{2+} induces an irreversible inhibition.

3- N-acetylglucosamine seems to be a key molecule in the process of germ tube formation when other sugars are used as a carbon source.

4- Glycosylation with N-acetylglucosamine and tyrosine phosphorylation of several proteins are detected during growth and myceliation of C. albicans, such biochemical events are regulated by Ca^{2+} and Mg^{2+}.

5- GRB2 and SOS (CDC25 homolog), are two proteins involved in cell growth and differentiation of eukaryotic cells, they also seem to play a role in the growth of C. albicans and S. cerevisiae.

HSP12 gene expression in *Saccharomyces cerevisiae*: monitor of osmotic stress

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Exposure of yeast cells to high external osmolarities evokes the socalled osmostress response. Most likely, both osmoregulatory and general stress reactions are involved in this poorly understood process. Part of the response aims at raising the internal osmotic potential, i.e. the production of osmolytes like glycerol, and exclusion of toxic solutes. In addition, heat shock proteins and trehalose are synthesized, to protect cellular components and mediate repair and recovery. We use the gene encoding the small heat shock protein Hsp12 as a model for investigating osmostress-induced changes in gene expression in *S. cerevisiae*. Hsp12, one of the two small heat shock proteins of *S. cerevisiae*, accumulates in response to osmotic and heat stress and upon entry of a yeast culture into stationary phase. This small Hsp displays the strongest induction upon osmotic stress of all yeast proteins, as can be judged from comparative 2D analysis of [3H]leucine-labeled protein extracts. In collaboration with Dr. P. Meacock, Univ. of Leicester, UK, we initiated the dissection of the *HSP12* promoter region in order to identify *cis*-acting elements and *trans*-acting factors involved in the regulation of this osmo-responsive gene. For this purpose, a DNA fragment containing 3300 bp of the *HPS12* 5'-flanking region was cloned from a yeast genomic DNA library and placed immediately upstream of the *GUS* (β-glucuronidase) reporter gene. 5' deletions were generated and the transcriptional activity of the resulting *HSP12-GUS* gene fusions was determined. Data obtained so far suggest that two upstream activating sequences (UAS) contribute to the induction of *HSP12* upon osmostress, whereas at least two repressor sites (URS) are needed to prevent transcription of the HSP12 gene under non-stressful conditions. Both positive and negative cis-acting elements were mapped in the most proximal 600 bp of the *HSP12* 5'-flanking region. Furthermore, we show that the Heat Shock Factor (Hsf) apparently does not play a major role in the activation of the *HSP12* gene either upon osmotic or heat stress, since similar mRNA levels accumulate in stressed *hsf1-m3* cells at the restrictive temperature, as compared with an isogenic wild-type strain. However, mutations in the Pbs2-Hog1 pathway do affect the induction of the *HSP12* gene in osmo-shocked cells. In order to shed light on the possible function of *HSP12* and *HOG1* gene products and their effect on the expression of other stress-induced genes, we overexpressed the *HSP12* and *HOG1* genes and examined the osmotolerance acquisition as well as the expression of the *HSP12*, *HSP26*, and *HSP82* genes in these yeast strains.

FEATURES OF Slt2p (Mpk1p) FUNCTION IN YEAST CELL INTEGRITY.

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Protein kinases are components of a number of signal transduction pathways involved in intracellular signal transmission. In last years, some biochemical and genetic studies have elucidated a cascade of three sequentially activating protein kinases that act as a conserved functional module in a wide variety of signal transduction pathways in diverse organisms. MAP kinases are the last elements of this module of protein kinases, participating as key molecules in the signalling process. Among others, a *S. cerevisiae* MAPK cascade essential for cell integrity, functions in a signal transduction pathway mediated by protein kinase C. Some screens and epistasis experiments have been useful to elucidate the steps downstream of Pkc1p, that include the sequentiall participation of Bck1p(Slk1p), Mkk1p/Mkk2p and the MAP kinase Slt2p (Mpk1p). By using the "two hybrid" system we have demonstrated that Slt2p interact *in vivo* with Mkk1p and Mkk2p.

SLT2 gene was isolated in our laboratory by its capacity to complement the autolytic phenotype of *lyt2* mutants. We have both rescued and amplified by PCR the *SLT2* allele of several *lyt2* strains. The recovered alleles were non functional as they were unable to complement the lytic phenotype of *slt2Δ* and *lyt2* strains. Nucleotide sequencing have allowed us to identify the mutation which abolish *SLT2* function: an AT to GC change determined a non-conserved substitution of aspartic acid for the glycine at position 35 of the amino acid sequence. Gly35 is the third glycine of a glycine cluster (Gly-X-Gly-X-X-Gly), a conserved region in protein kinases and other nucleotide binding proteins. We have also shown by Western blot that this mutation did not affect the structure of the mutant protein. These evidences show that *lyt2* mutation is allelic to *SLT2* gene and that this glycine is essential for Slt2p activity.

Cloning and functional characterization of *MKC1*, a *Candida albicans* MAP kinase related to cell growth and cell wall integrity

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Recent work in *Saccharomyces cerevisiae* has led to the identification of different signal transduction pathways, among them, the *PKC1*-mediated pathway. The fact that mutants defective in elements of this last pathway display a lytic phenotype (which is at least partially complementable upon addition of osmotic stabilizers to the medium) suggests a role for this pathway not only in cell growth but also in the construction of stable cell wall. In all these cases, phosphorylation seems to be the mechanism used by the cell to transmit the signal to the transcription machinery, thus allowing the generation of different cellular responses.

In order to obtain information concerning the role of this cascade in pathogenic yeasts, we have undertaken the isolation of the *C. albicans* homolog of the *S. cerevisiae* MAP kinase coded by the *SLT2* gene. A search for complementation of the lytic phenotype of *S. cerevisiae slt2Δ* mutants using a episomal genomic library yielded a DNA fragment able to partially complement the mutant phenotype, in which a single ORF of 502 aa (predicted Mw 58.32 kDa) was found. The gene was named *MKC1* (Map Kinase of *Candida*) and belongs to the MAP kinase family. Mkc1p is 55% identical to Slt2p, 45.8% to Fus3p, 36.8% to Hog1p and 42.3% to Cek1p, a *Candida* Fus3p/Kss1p homolog. Due to the natural diploidy of this yeast and lack of sexual cycle, the disruption of *MKC1* in *C. albicans* was achieved by homologous recombination in a two step procedure using *URA3* as selectable marker. *mkc1Δ* homozygous strains display an osmotic remediable caffeine hypersensitivity and thermosensitivity at 42°C, as well as reduced viability in stationary phase, phenotypic traits also found in *S. cerevisiae slt2* mutants. In addition, some novel features are found: first, the tolerance to thermal shocks at 55°C is much more limited when compared to wild type strain; second, they are more sensitive to cell wall digestion, indicating a probable defect in cell wall structure; finally, they display some characteristic morphological changes which result in different colony morphology in high salt media and ability to undergo the morphological transition. These results not only suggests the presence of the *PKC1*-mediated pathway in this organism but also evidences the key role played by this MAP kinase in normal cell growth.

Several Pathways Regulate Potassium Transport in *Saccharomyces cerevisiae.*

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Yeast cells accumulate high concentrations of potassium to fulfill their cellular requirements. The regulation of potassium transport is a complex process in which factors such as membrane potential, cellular pH or the potassium content of the cell, are important regulatory factors in *Saccharomyces cerevisiae* (1).

Our group has recently shown that, in addition to the above mentioned factors, the presence of glucose or other fermentable sugar in the medium activates the process of potassium transport (2). It increases the velocity of transport, without affecting the affinity of the carrier and requires the sugar to be transported inside of the cell. The presence of a functional kinase able to phosphorilate the sugar is also required but, apparently, not further metabolism of the sugar it is necessary, since 2-deoxiglucose also increases the velocity of potassium transport.

Recent results suggest that a new pathway, similar to the PIP_2 pathway of mammalian cells, may be involved in the regulation of potassium movements in yeast. According to this idea, very low concentrations of calmidazolium and compound 48/80 which inhibit calmodulin dependent processes and protein kinase C in mammalian cells, inhibit almost completely potassium transport in *S. cerevisiae*. This inhibition occurs, both in wild type cells and in the different potassium transport mutants available, suggesting that it really affects a different process. We have isolated several mutants resistant to Calmidazolium and to compound 48/80. They show higher capacity to transport potassium than the wild type.The characterization of these mutants from a molecular biologie point of view is now in progress.

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CHARACTERIZATION OF THE YEAST (1→6)-β-GLUCAN BIOSYNTHETIC COMPONENTS, Kre6p AND Skn1p; AND GENTETIC INTERACTIONS BETWEEN THE *PKC1* PATHWAY AND EXTRACELLULAR MATRIX ASSEMBLY.

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A characterization of the *S. cerevisiae KRE6* and *SKN1* gene products extends previous genetic studies on their role in (1→6)-β-glucan biosynthesis. *KRE6* and *SKN1* are predicted to encode homologous proteins that participate in assembly of the cell wall polymer (1→6)-β-glucan. *KRE6* and *SKN1* encode phosphorylated integral-membrane glycoproteins, with Kre6p likely localized within a Golgi subcompartment. Deletion of both these genes is shown to result in a dramatic disorganization of cell wall ultrastructure. Consistent with their direct role in the assembly of this polymer, both Kre6p and Skn1p possess C-terminal domains with significant sequence similarity to two recently identified glucan-binding proteins.

Deletion of the yeast protein kinase C homolog, *PKC1*, leads to a lysis defect. Kre6p when even mildly overproduced, can suppress this *pkc1* lysis defect. When mutated, several *KRE* pathway genes and members of the *PKC1*-mediated MAP kinase pathway have synthetic lethal interactions as double mutants. These suppression and synthetic lethal interactions, as well as reduced β-glucan and mannan levels in the *pkc1* null wall, support a role for the *PKC1* pathway functioning in cell wall assembly. *PKC1* potentially participates in cell wall assembly by regulating the synthesis of cell wall components, including (1→6)-β-glucan.

Functional analysis of *CAL1* gene of *Saccharomyces cerevisiae.*

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The gene *CAL1* of *S.cerevisiae* encodes for the chitin synthase III activity. This activity is the responsible of the majority of chitin biosynthesis in this yeast. However this gene shows only limited homology to other chitin synthases described so far. This study focus on the functional characterization of *CAL1* gene and its product.

CAL1 has not significant homology to other chitin synthases in their carboxy-terminal region, that result could suggest that this region is highly important for the specific function of this gene.We are analyzing the function of this carboxy region through in vitro mutagenesis. Our results clearly show that this region is essential for function. The phenotype of different truncated proteins have been analyzed in vivo by measuring chitin biosynthesis, resistance to calcofluor and pattern of chitin deposition. Preliminary data indicated that this domain could be implicated in mediating protein/protein interactions. We are currently analyzing the effect of these truncated proteins in the chitin synthase III activity.

In addition, using PCR amplification, we have just shown (Roncero *et al.*, submited) that *CAL1* is conserved among yeast and fungi. The degree of conservation in the amplified region is very high, suggesting that the function of this gene is highly conserved.

Our results will be discussed in relationship with the role of *CAL1* and homologous genes in chitin synthase III activity and in the biosynthesis of chitin in yeasts and fungi.

lyt1-1: a _cdc_ like _Saccharomyces cerevisiae_ mutant showing alteration in the budding pattern, autolysis in the mitotic cycle and impaired sporulation.

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We have described *lyt1-1* mutation in *S. cerevisiae* as a pleiotropic one that determines several phenotypic traits. Among then are a thermosensitive arrest of the mitotic cycle that occurs in cell with buds of the size of a daughter cell, a thermosensitive cell lysis, a thermosensitive alteration of the budding pattern that changes from axial to polar in haploid strains and a lack of sporulation capacity of homozigous diploids (1).

Transformation of this mutant with a genomic library bound to a centromeric vector permitted the isolation of a clon that was able to complement the aforementioned phenotypical traits, and carried genes *CDC15* and *NPK1*. Gene *CDC15* complements autolytic and Cdc phenotypes, but needs the presence of gene *NPK1* to accomplish sporulation. These results may indicate the existence, in mutant Lyt1, of two mutations in contiguous genes (*CDC15* and *NPK1*); in spite of the fact that previous experiments (2) seem to indicate the existence of a single mutation. Alternatively, gene *NPK1* may be needed for sporulation only in a *lyt1-1* background.

CDC15 encodes a protein-kinase implied in the deactivation of the complex CDC28-cyclins, and it is necessary to surpass the "END" point of the cell cycle (3).

On the other hand, gene *SPO12* was isolated as a suppressor of Lyt1 mitotic phenotypical traits (1), and subsequent experiments have demonstrated that it also suppress mutant Cdc15 phenotype. Two other suppressor genes were also cloned using cDNA human and fission yeast libraries, and *lyt1* as the recipient strain. Both of the clones isolated were only able to complement the defect in sporulation.

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FAR1 is required for polarization towards a mating partner during conjugation in *S. cerevisiae*.

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S. cerevisiae exhibits polarized cell growth during two phases of its life cycle: bud emergence and mating. This polarity is manifested in the orientation of the cytoskeleton and the localization of secretion. We are interested in identifying genes required for a cell to locate its partner during mating.

We are using two assays to analyze a collection of mutants which are good candidates for cells defective in finding a mating partner. These mutants exhibit normal cell cycle arrest and shmoo formation in the presence of pheromone but are defective in mating. The ability of these mutants to polarize their growth towards a source of pheromone is being determined using a direct microscopic assay in which MAT a cells are presented with a gradient of alpha factor (Segall, 1994).

A second assay for the ability to locate a mating partner determines the mating frequency of each mutant strain under specific conditions. This experiment quantitates the mating of a MAT a cell to a wildtype MAT alpha partner in the presence of a vast excess of alpha factor. It is widely accepted that a cell experiences a gradient of pheromone during conjugation and uses this gradient to locate its mating partner. If a wildtype cell mates in an excess of pheromone, a dramatic reduction in its ability to mate is seen (approximately 100 fold). We think this is because the added pheromone disrupts the cell's ability to locate its mating partner since the pheromone gradient is destroyed. Any mutant unable to locate its mating partner should not display a further reduction of mating frequency in this assay. The advantage of this assay is that much larger numbers of cells can be analyzed.

Using these two assays we have identified four mutants which cannot polarize towards a source of pheromone. All of these mutants carry alleles of *FAR1* effecting the C terminus of the protein. It has long been known that the C terminus of Far1p is required for mating, independent of the protein's role in cell cycle arrest. (In fact these mutants all cell cycle arrest normally.) These results suggest that Far1p functions during conjugation to locate the mating partner spatially. We are interested in pursuing how it does this.

List of Invited Speakers

Workshop on

SIGNAL TRANSDUCTION PATHWAYS ESSENTIAL FOR YEAST MORPHOGENESIS AND CELL INTEGRITY

List of Invited Speakers

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