Overexpression of the G₁-cyclin Gene CLN2 Represses the Mating Pathway in Saccharomyces cerevisiae at the Level of the MEKK Ste11*

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Basal and induced transcription of pheromone-dependent genes is regulated in a cell cycle-dependent way. FUS1, a gene strongly induced after pheromone treatment, shows high mRNA levels in mitosis and early G₁ phase of the cell cycle, a decrease in G₁ after START and again an increase in S phase. Overexpression of CLN2 was shown to repress the transcript number of pheromone-dependent genes (1). We asked whether the activities of components of the mating pathway fluctuate during the cell cycle. We were also interested in determining at what level Cln2 represses the signal transduction machinery. Here we show that the activity of the mitogen-activated protein kinase Fus3 indeed fluctuates during the cell cycle, reflecting the oscillations of the gene transcripts. CLN2 overexpression represses Fus3 kinase activity, independently of the phosphatase Msg5. Additionally, we show that the activity of the MEK Ste7 also fluctuates during the cell cycle. Increased Cln2 levels repress the ability of hyperactive STE11 alleles to induce the pathway. G protein-independent activation of Ste11 caused by an rga1 pbs2 mutation is resistant to high levels of Cln2 kinase. Therefore our results suggest that Cln2-dependent repression of the mating pathway occurs at the level of Ste11.

Mating between cells of the two opposite haploid cell types of Saccharomyces cerevisiae requires recognition of the mating partner through secreted pheromones. In response to pheromone both mating partners arrest their cell cycle in G₁ (2, 3) and induce a set of pheromone-dependent genes (4). They undergo morphological changes (shmoo formation), which enables two cells of opposite mating type to fuse and form a diploid zygote. Mating is restricted to a short period in the G₁ phase of the cell cycle (5–8). Cells which have passed a point called START in G₁ are committed to undergo a new cell cycle and cannot respond to mating pheromone until they reach G₁ again (9, 10). Transition through START is mediated by the function of the G₁ cyclins Cln1, Cln2, and Cln3, which are the regulatory subunits of the Cdc28 kinase (11–15). In response to mating pheromone the activity of the Cdc28-Cln kinase gets inhibited, so that the cell cannot pass START (16). Mating pheromone induces a MAPK pathway which ultimately results in the phosphorylation of the putative Cdk inhibitor (cyclin-dependent kinase inhibitor) Far1 (17, 18). Phosphorylation of Far1 causes its association with the Cdc28-Cln1 and Cdc28-Cln2 kinases and thereby inhibits their ability to drive the cell through START (17–19). Both mating partners are arrested in the same stage of the cell cycle when they fuse. This mechanism ensures the correct ploidy of the zygote.

Transcription of pheromone-inducible genes such as FUS1, STE2 (1, 19), SST2, STE12, and MFA2 (20) has been shown to fluctuate during the cell cycle. It reaches its maximum during late mitosis and early G₁, decreases drastically in late G₁ around START, and increases again in the G₂ phase of the cell cycle (1, 20). The transcript of FAR1, which encodes a putative cyclin-dependent kinase inhibitor, also fluctuates during the cell cycle, but in contrast to other pheromone-inducible genes, its transcription in G₂/M is Mcm1-dependent (20). The stability of the Far1 protein is also regulated during the cell cycle. Far1 protein can be detected only in early G₁ cells and is rapidly degraded in other stages of the cell cycle (10). This ensures that the cell cycle arrest in response to pheromone occurs in early G₁ only.

The cell cycle regulated restriction of pheromone-dependent gene transcription may reflect an important mechanism ensuring that the responsiveness of the cell to mating pheromone is maximal in early G₁. The decrease of pheromone-dependent gene transcription in late G₁ correlates with an increase in the appearance of G₁ cyclin. Overexpression of CLN2 represses FUS1 transcription (1). Preliminary epistasis experiments demonstrated that the repression occurs downstream of the receptor (1) and that it involves neither Sst2 (implicated in recovery from pheromone-induced G₁ arrest) nor the carboxy-terminal part of the pheromone receptor (implicated in desensitization).

Potential targets of Cln2-mediated repression are the components of the pheromone-induced MAPK pathway. This signal transduction pathway has been the focus of several reviews (4, 21, 22). In short, activation is mediated by the binding of pheromone to a seven-transmembrane receptor coupled to a heterotrimeric G protein. Upon pheromone induction Gα dissociates and releases Gβγ (23–27). Gβγ transmits the signal to the Ste20 kinase (28, 29) by an as yet unknown mechanism involving the GTPase Cdc42, the guanine nucleotide exchange factor Cdc24 (30, 31) and the GTPase-activating protein Rga1 (32). Downstream of Ste20 the sequential activation of several protein kinases, tethered together by the scaffold protein Ste5, propagates the signal (28). These kinases belong to the family

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; MEKK, MEK kinase; MOPS, 4-morpholinepropanesulfonic acid.
of highly conserved MAPK pathways (21) and function in the linear order Ste11, Ste7, and Fus3/Kas1. The MAPKs Fus3 and Kas1 are thought to activate the transcription factor Ste12, which mediates the transcriptional induction of pheromone-dependent genes (33–35).

In this work we investigate which components of the pheromone-induced signal transduction pathway are regulated in a cell-cycle-dependent way. We show that the activity of the MAPK Fus3 fluctuates during the cell cycle. Furthermore, Fus3 kinase activity is repressed by overexpression of CLN2. The repression occurs independent of the function of the phosphatase MSG5, which dephosphorylates Fus3 (36). The activity of the MEK Ste7 was shown to respond to the cell cycle as well. By the use of hyperactive alleles of Ste11 and a deletion of RGA1, we conclude that CLN2 represses the mating pathway at the level of Ste11. The studies in this work represent an important contribution to the understanding of the regulation of a signaling cascade by the cell cycle and the phenomenon described here may be relevant in the regulation of MAPK pathways in higher eukaryotic cells.

MATERIALS AND METHODS

Yeast Strains—The *S. cerevisiae* strains utilized in this study are listed in Table I. Standard yeast techniques were used as described in Sherman et al. (37). YEP (complete) medium, minimal synthetic medium, and supplements are described by Hicks and Herskowitz (38). Yeast transformations were performed as described by Ito et al. (39) and Beggs (40), or by one-step transformation (41). Induction of *S. cerevisiae* cells with pheromone required 0.3 μg/ml α-factor for *bar1* strains and 1 μg/ml for *BAR1* strains. Unless otherwise indicated cells were induced for 10 min with pheromone. For the expression of the galactosidase inducible GAL1–10 CLN2 construct cells were pregrown on YEP medium plus 2% raffinose, or, where indicated, glucose was added to a final concentration of 16 μM. Induction of the T7 promoter was induced for 10 min with pheromone. For the expression of the protein the protein are described in Peter et al. (17). Fus3-R152 was expressed as a glutathione S-transferase fusion protein from the plasmid GA1944 in *E. coli* and purified as described

### Table I Yeast strains

| Strain | Relevant genotype | Source |
|--------|------------------|--------|
| SY1390 | MATa STE1-1 FY1::HIS3 leu2 ura3 trp1 his3200::ura3 pep4::ura3 can1 | Stevenson et al. (51) |
| SY1865 | MATa STE1-6 FY1::HIS3 leu2 ura3 trp1 his3200::ura3 pep4::ura3 can1 | Stevenson et al. (51) |
| SY1866 | MATa STE1-6 FY1::HIS3 leu2 ura3 trp1 his3200::ura3 pep4::ura3 can1 | Stevenson et al. (51) |
| SY1969 | STE1-6 FY1::HIS3 leu2 ura3 trp1 his3200::ura3 pep4::ura3 can1 | Stevenson et al. (51) |
| KS114  | MATa trp1 leu2 ura3 his4 | J. Pringle |

Plasmid Constructions—Standard DNA manipulations were performed according to Sambrook et al. (42) and Ausubel et al. (43). In Table II the plasmids used in this study are listed. The GAL1–10 CLN2 plasmid K2573 was cut with EcoR I and integrated into the genome. The integration was checked by Southern blotting.

Expression and Purification of Far1 and Fus3-R152 in Escherichia coli—Far1 was expressed in *E. coli* from the plasmid GA1896, which contains the NH2-terminal fragment of Far1 (amino acids 50–301) under the control of the isopropyl β-D-thiogalactopyranoside-inducible T7 promoter. Expression and purification of the protein are described in Peter et al. (17). Fus3-R152 was expressed as a glutathione S-transferase fusion protein from the plasmid GA1944 in *E. coli* and purified as described in Errede et al. (44).

**Fus3 Kinase Assays**—Fus3 kinase assays were done as described in Peter et al. (17), except that the reactions were done in 6 μl of HBII buffer, where 3 μl of [γ-32P]ATP (6000Ci/mmol), 0.5–2 μl (0.1 μg/μl) of Far1 substrate, and 25 mM MOPS, pH 7.2, up to a final volume of 16 μl were added. The reactions were carried out at 30 °C for 30 min.

**Ste7 Kinase Assays**—The strains used contain the *Myc* epitope-tagged wild-type protein (pNC318) or an inactivated version of Ste7 (pNC318-R220) on a centromeric plasmid under the control of the CYC1 promoter (45). 70-ml cultures were grown to an OD600 of around 0.8 in YEP plus 2% sucrose. cdc15-2 and cdc4-1 cells were arrested as described. Unless otherwise indicated everything was done at 4 °C.

Cells were harvested by centrifugation, washed with kinase extract buffer (KEB) (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM sodium fluoride, 30 mM Na2HPO4, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 40 μM aprotinin, and 20 μg/ml leupeptin), and resuspended in 200 μl of breakage buffer (KEB without sodium fluoride and Na2HPO4). Cells were lysed by vortexing them with glass beads 3 times for 4 min. The extracts were cleared by centrifugation once for 5 min and twice for 15 min at 14,000 rpm. The protein content was determined using the Bio-Rad protein assay as recommended by the manufacturer. 300 μg of protein extract were used for the immunoprecipitation. Ste7 was precipitated with approximately 10 μg of anti-Myc 9E10 antibody for 1.5 h. 20 μl of protein A-Sepharose beads in a 1:1 suspension (preincubated in breakage buffer) were added. After 1 h incubation the beads were washed 5 times in breakage buffer, and 3 times in HEPES, 25 μl, pH 7.5. For the kinase assay, 6 μl of kinase extract buffer (25 mM HEPES, pH 7.5, 15 mM MgCl2, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM sodium fluoride, 30 mM Na2HPO4, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 40 μg/ml aprotinin, and 20 μg/ml leupeptin) were added to the beads. 0.5 μg of *E. coli* purified Fus3-R152 as a substrate, 0.2 μl of [γ-32P]ATP (10 μCi/μl), 1 μl of HEPES, 250 mM, 2 μl of 1 mM ATP, and H2O up to a final volume of 20 μl were added. The kinase assay was incubated at 30 °C for 30 min. The reaction was stopped by adding SDS sample buffer (46) and boiling the extracts for 4 min. Phosphorylation of the substrate was detected by SDS-polyacrylamide gel electrophoresis analysis on a 9.5% polyacrylamide (28:2, acrylamide-bisacrylamide) gel (46).

RNA Preparation, Northern Blot Analysis—Yeast total RNA preparation was performed as described by Cross and Tinklenberg (47). RNA was separated on formaldehyde-agarose gels and transferred to Boehringer Mannheim nylon membranes (as recommended by the manufacturer). Northern blots were processed as described by Price et al. (48). The DNA fragments used as probes were labeled by random priming and isolated as follows: SST2, a ClaI/HpaI fragment was cut out from the SST2 coding region; CMD1, with the oligos 5-GTCCTC-
CAATCTTACCGAG-3' and 5'-TTACAAGTAGAATCTTAGATAGAG-3' the CMD1 coding region was isolated by PCR from genomic DNA; CLN2, a SacII/ClaI fragment was cut out from the CLN2 coding region; CDC28, an EcoRI/PstI fragment from the plasmid pBR322, containing a GAL1–10 CDC28 construct (by A. Amon) was isolated; FUS1, the HindIII/SalI fragment of the FUS1 coding region was isolated; STE5, a HindIII fragment from the STE3 coding region was isolated; ACT1, a XhoI/KpnI fragment from the ACT1 coding sequence.

FACScan Analysis—DNA content of propidium iodide-stained cells was measured on a Becton-Dickinson FACScan as described by Lew et al. (49) and Epstein and Cross (50).

cdc15–2 and cdc4–1 Cell Cycle Arrest—Cells were grown at permissive temperature at 24 °C in the appropriate medium from OD600 0.2 to 0.8. The cultures were diluted to OD600 0.4 and arrested for 3–4 h at 37 °C. The arrest was checked by microscopic examination and determination of the budding index.

cdc15–2 Release Experiments—cdc15–2 cells were arrested as described above. The cultures were released by diluting them with ice-cold YEPD to OD600 0.5 and further cooling them down on ice till the temperature was 24 °C. They were released at 24 °C. Aliquots were taken at the indicated time points for kinase assays, FACS analysis, RNA preparation, protein extracts for Western blots, and for the determination of the budding index.

RESULTS

Fus3 MAPK Activity Fluctuates during the Cell Cycle—The transcripts of pheromone-dependent genes fluctuate during the cell cycle (1, 19, 20). To determine whether the transcriptional fluctuations are the consequence of differences in the activity of the MAPK Fus3, we expressed FUS3 constitutively from the TP11 promoter to examine kinase activity independently of its transcriptional regulation during the cell cycle. Kinase assays from distinct cell cycle arrested cells were performed with a Myc epitope-tagged Fus3. Fus3 was immunoprecipitated with 9E10 anti-Myc monoclonal antibody. Cells harboring a temperature-sensitive mutation in the CDC15 or the CDC4 gene were arrested at the restrictive temperature in late mitosis or late G1, respectively. In vitro Fus3 kinase assays using bacterial recombinant Far1 as a substrate were performed under pheromone-induced conditions (Fig. 1A) and uninduced conditions (data not shown). According to the fluctuations observed for pheromone-dependent genes, Fus3 MAPK activity was expected to be high in cells arrested in late mitosis, and low in cells arrested in late G1. As can be seen in Fig. 1A, Fus3 kinase activity shows the same cell cycle-dependent pattern as described for the FUS1 transcript (1). Fus3 activity is high in cdc15–2 arrested cells (lanes 1 and 2) and low in cdc4–1 arrested cells (lanes 4 and 5).

To further analyze the cell cycle-dependent regulation of basal Fus3 kinase activity we performed cdc15–2 release experiments in the absence of pheromone. The cultures were arrested at restrictive temperature in late mitosis and released from the block at permissive temperature. The arrest was checked by microscopic examination and determination of the budding index (data not shown). At the indicated time points, samples were taken for kinase assays (Fig. 1B) and Northern analysis (Fig. 1C). The activity of Fus3 was examined by in vitro kinase assays. As seen in Fig. 1B the activity of Fus3 shows the same cell cycle-dependent regulation which had been observed for the transcripts of mating dependent genes.

Differences in the activity of Fus3 could have been due to differences in Fus3 protein levels. To rule out this possibility, Western blot analysis was performed. Aliquots from the cdc15–2 release experiment in Fig. 1, B and C, were used for the preparation of cell extracts. The Western blot was probed with anti-Fus3 and anti-Cdc82 polyclonal antibodies. The Fus3 protein concentration stays constant throughout the cell cycle and is not responsible for the fluctuations in kinase activity or in expression of pheromone-dependent genes (data not shown).

A second release experiment to study the timing of Fus3 activity during the cell cycle was performed. The release was checked by FACScan analysis (Fig. 1E). Fus3 activity and CLN2 mRNA fluctuate antagonistically (Fig. 1D). These data are in accordance with previous studies showing that the FUS1 and CLN2 transcripts fluctuate antagonistically (1). Basal and pheromone-induced activity of the MAPK Fus3 are cell cycle-regulated, but the protein levels of Fus3 stay constant. The activity of the MAPK Fus3 reaches its maximum in late mitosis and early G1, drops after START, is low in late G1, and increases again in S phase. The pattern of its cell cycle regulation is the same as observed for the FUS1 transcript and reaches its maximum when CLN2 levels are low.

Overexpression of CLN2 Represses Fus3 Kinase Activity—Overexpression of the G1 cyclin genes CLN1 and CLN2 represses transcription of FUS1 (1). To see whether CLN2 overexpression reduces Fus3 kinase activity, CLN2 was overexpressed from the GAL1–10 promoter in cycling cells and Fus3 activity was examined be in vitro kinase assays. Myc epitope-tagged Fus3, expressed from the TP11 promoter, was immunoprecipitated with anti-Myc 9E10 antibodies. We confirmed that the steady state protein levels of Fus3M in these strains are not affected by overexpression of CLN2 using Western blot analysis with Fus3 specific polyclonal antibodies (data not shown). A bacterial recombinant NHEterminal fragment of Far1 served as a substrate for Fus3 in immunoprecipitation assays. Fig. 2A shows that Fus3 activity is strongly repressed in cells overexpressing CLN2 from the GAL1–10 promoter (lanes 2 and 4) compared with cells where the promoter is not induced (lanes 1 and 3). This holds true not only for basal levels of Fus3 activity but also for pheromone-induced levels (lanes 3 and 4).

To confirm that the down-regulation of Fus3 activity was due to Cln2 expression and not to distinct growth conditions as a result of different carbohydrate sources we compared strains harboring the GAL1–10 CLN2 construct and the empty vector alone, respectively, in a Northern blot experiment. The same levels of the pheromone-inducible genes SST2 and STE3, irrespectively of the source of carbohydrates used in the media, were observed (Fig. 2C, lanes 1 and 2 for STE3, lanes 7 and 8
for SST2). At the same time, cells harboring the GAL1–10 CLN2 construct repress SST2 and STE3 transcription on galactose containing medium (Fig. 2C, compare lanes 3 and 4 for STE3, compare lanes 5 and 6 for SST2), as it was already shown for FUS1 transcription (1). Assuming that the transcription of SST2 and STE3 reflects the kinase activity of Fus3, the experiment demonstrates that repression of Fus3 activity occurs because of elevated Cln2 levels and not because of different carbohydrate sources.

CLN2-dependent Repression of Fus3 Activity Does Not Require Msg5 Function—Either positive or negative factors could mediate a cell cycle-dependent fluctuation in Fus3 activity. It was demonstrated by Doi et al. (36) that the protein phosphatase Msg5 dephosphorylates Fus3 and thereby inactivates it. Loss of Msg5 activity causes an increase in Fus3 activity. Repression of Fus3 activity through overexpression of CLN2 could be regulated by increased Msg5 activity. We wanted to elucidate the potential role of Msg5 in mediating the downregulation of Fus3 activity in cells with elevated Cln2 levels.

For this purpose, the effect of CLN2 overexpression on Fus3 activity was examined in a MSG5 deletion strain. Fus3 kinase assay were performed as described above. Fig. 3 shows that repression of Fus3 by Cln2 does not require the Msg5 phosphatase (compare also with Fig. 2) and therefore must work through a different mechanism (Fig. 3, lanes 1 and 2 (no a-factor treatment) and lanes 3 and 4 (a-factor treatment for 10 min)). The studies described above indicate that the cell cycle machinery represses the mating pathway upstream of the MAPK Fus3.

Ste7 MEK Activity Fluctuates during the Cell Cycle—Experiments were performed to see whether the activity of the MEK Ste7 is also cell cycle-regulated. Cells were arrested with either cdc15–2 or cdc4–1, and Ste7 kinase assays were performed under pheromone-uninduced conditions. Myc epitope-tagged Ste7 was expressed constitutively from the CYC1 promoter to avoid a potential cell cycle regulation of the STE7 transcript. As a control wild-type cells harboring the same STE7 construct and cells with a construct containing a mutated version of
STE7 were used. These cells were treated with pheromone. Fig. 4 shows that the activity of Ste7 is high in late mitosis (cdc15–2 arrest, lane 4) and low in late G1 (cdc4–1 arrest, lane 3), as is the case for Fus3.

Epistasis Experiments with Hyperactive Alleles of STE11—To further see which components in the pathway are regulated by the cell cycle, studies with hyperactive alleles of the MEKK Ste11 were done. STE11–1 and STE11–4 hyperactive alleles induce the pheromone pathway independent from pheromone treatment and independent from G protein function (51). In this case, downstream targets of Ste11, like the MEK Ste7 and the MAPK Fus3, are induced and activate pheromone-dependent genes. Down-regulation of Fus3 activity or pheromone-dependent transcripts by overexpression of CLN2 in cells induced by an hyperactive Ste11 allele would indicate that Cln2 regulates the pathway through Ste7, Ste11, or an event that controls the activity of the hyperactive Ste11 protein.

Fig. 5A shows that overexpression of CLN2 represses the increased transcription of SST2 in strains harboring the hyperactive STE11 alleles (compare lanes 3 and 4 (STE11–4 allele) and lanes 5 and 6 (STE11–1 allele) with lanes 1 and 2 (wild-type allele)). The transcription of another pheromone-inducible gene, STE3, was also repressed (data not shown). Fus3 kinase assays were performed as described above and demonstrate that the activity of Fus3 in cells harboring STE11–1 is still down-regulated by overexpression of CLN2 (Fig. 5B, compare lanes 3 and 4, and the wild-type GA145, lane 7).

**SST2, STE3, CDC28, controls, glucose was added (lanes 2, 3, 6)**
Galactose was added for 2.5 h to induce the GAL1–10 CLN2 overexpression. All strains used contain the CLN2::URA3 rga1 KW19 (wild-type kinase assay as described in Fig. 1 was performed. The strains used are (STE11–4 allele (KW20), and the STE11 gene in a wild-type STE11–1 allele (KW21), the STE11 allele (KW19), the STE11 allele), both harboring the plasmid GA1903 (FUS2, Muc epitope-tagged, under the control of the TP11 promoter). Galactose was added where indicated (lanes 2 and 4) for 2.5 h. To the controls, glucose was added (lanes 1 and 3). C, RNA was prepared from the following strains: BSY187 (ste4), BSY193 (ste4 rga1 pbs2), and KW24 (ste4 rga1 pbs2 GAL1–10 CLN2::URA3). Where indicated, the strains harbor the GAL1–10 CLN2::URA3 integration (lanes 5–10). RNA was prepared from 2.5 h galactose-induced cells (lanes 2, 4, 6, 8, and 9) and from controls, which were grown in raffinose-containing medium (lane 10), or where glucose was added instead (lanes 1, 3, 5, and 7). The Northern blots were probed with SST2, CMD1, STE3, CDC28, and CLN2.

Fig. 5. A and B, CLN2 represses STE11 hyperactive alleles whereas rga1-dependent induction of the mating pathway is not repressed by CLN2 overexpression. All strains used contain the GAL1–10 CLN2::URA3 integration. A, RNA was prepared from strains containing the wild-type STE11 allele (KW19), the STE11–1 allele (KW21), the STE11–4 allele (KW20), and the STE11–1 allele in a ste4 background (KW24). Galactose was added for 2.5 h to induce the GAL1–10 promoter (lanes 2, 4, 6, and 8). To the controls, glucose was added (lanes 1, 3, 5, and 7). RNA blots were probed with SST2, CMD1, and CLN2. B, a FUS3 kinase assay as described in Fig. 1 was performed. The strains used are KW19 (wild-type STE11 allele) and KW21 (STE11–1 allele), both harboring the plasmid GA1903 (FUS2, Muc epitope-tagged, under the control of the TP11 promoter). Galactose was added where indicated (lanes 2 and 4) for 2.5 h. To the controls, glucose was added (lanes 1 and 3). C, RNA was prepared from the following strains: BSY187 (ste4), BSY193 (ste4 rga1 pbs2), and KW24 (ste4 rga1 pbs2 GAL1–10 CLN2::URA3). Where indicated, the strains harbor the GAL1–10 CLN2::URA3 integration (lanes 5–10). RNA was prepared from 2.5 h galactose-induced cells (lanes 2, 4, 6, 8, and 9) and from controls, which were grown in raffinose-containing medium (lane 10), or where glucose was added instead (lanes 1, 3, 5, and 7). The Northern blots were probed with SST2, CMD1, STE3, CDC28, and CLN2.

Fig. 6. CLN2-dependent repression of the mating pathway does not require CLA2 and BEM1. RNA was prepared from K1107 (cla2::LEU2), KS116 (bem1::LEU2), and their isogenic wild-types K1107 (lanes 1 and 2) and KS114 (lanes 5 and 6). The cells were grown on YEP medium plus glucose. Where indicated, strains contain the plasmid GA1855 (CLN2 under the control of the TP11 promoter on a centromeric plasmid) (lanes 1, 3, 5, and 7). The Northern blot was probed with FUS1, CLN2, and CMD1.

and 8) and STE3 (data not shown) was examined. Cln2 represses the mRNA levels of SST2 and STE3. The Cln2-dependent down-regulation of the mating pathway works independently of the G protein. It can be concluded that Cln2 represses the mating pathway downstream of the G protein.

Activation of the Pathway Due to a Deletion in RGA1 Is Not Repressed by Overexpression of CLN2—To further see at what point of the signaling cascade Cln2 represses the mating pathway a strain carrying a deletion in the RGA1 gene was used. RGA1 was first characterized by Stevenson et al. (32) as a putative GTPase-activating protein for Cdc42 (32). Deletion of rga1 increases signaling of the pheromone pathway, an effect which is enhanced by a PBS2 deletion. A deletion in RGA1 and PBS2 activates the pheromone-dependent signal transduction pathway independently of the G protein, but requires function of Ste20 and Ste11. It functions upstream, or at the level of Ste11 (32). Fig. 5C shows the transcriptional level of SST2 and STE3 in strains deleted for STE4 which normally has very low basal activity of the pheromone-dependent pathway. Indeed, in the single mutant ste4 strain, hardly any SST2 transcription can be detected (Fig. 5C, lanes 1 and 2). In a ste4 rga1 pbs2 strain the pathway is more active (Fig. 5C, lanes 3, 5, 7, and 10). The activation is comparable to basal levels in a wild-type strain (32).

We wanted to know whether CLN2 overexpression represses transcription of SST2 and STE3 in a ste4 rga1 pbs2 mutant: CLN2 overexpressed from the GAL1–10 promoter is not able to repress the activation of the mating pathway due to an rga1 deletion (Fig. 5C, compare lanes 5 and 6, lanes 7 and 8, and lanes 9 and 10).

The following conclusions can be drawn from the experiments described above: Cln2-dependent repression does not seem to occur downstream of Ste11, as this would enable Cln2 to repress the signal obtained in a ste4 rga1 pbs2 strain. Cln2-dependent repression does not require function of the G protein, as the hyperactive STE11–1 allele is down-regulated in a ste4 strain. The repression seems to occur at the same level as Ste11. Possible targets left are, for example, the Cdc42 component, Ste5 and Ste20. Other potential targets are Bem1 (54) and Cla2 (55, 56), proteins involved in polarized growth, an event which is induced at the level of Cdc42.

Cln2-dependent Repression Does Not Require Bem1 and Cla2 Function—Bem1 was shown to be important for cell polarity during budding. This protein associates with Cdc42 (57). Bem1 also forms a complex with Ste20, Ste5, and actin and thus seems to be necessary for polarized rearrangement of the actin cytoskeleton prior to mating (54). We thought of Bem1 as a
Activation of bud emergence. For this reason a repression of the mating pathway by Cln2 could involve the function was found to be synthetic lethal with activation would prevent activation of Rsr1. Loss of Bem1 (60). Cla2 could therefore be a target for Cln2, whose manner (53). The formation of this complex might involve may form a complex with Rsr1-GTP at the presumptive bud localization and formation of buds and seems to be an essential part of the mechanism ensuring the accurate arrest in G1 in response to mating pheromone.

Candidate for Cln2-dependent repression of the mating pathway as it may act at the same level as STE11. Bem1 might have been either a direct target of Cln2 or mediator of Ste5 or Ste20 repression. To answer the question whether Bem1 was involved in the repression of the pathway by Cln2, the following experiment was performed: in a bem-1 strain CLN2 was over-expressed from the constitutive TPI1 promoter and the effect on FUS1 transcription was investigated. The bem-1 strain is temperature-sensitive. CLN2 repressed FUS1 mRNA accumulation on permissive (Fig. 6, lanes 5–8) and restrictive temperature (data not shown). We conclude that Bem1 is not necessary for Cln2-dependent repression of the mating pathway.

CLN2 codes for a GTPase-activating protein and is necessary for budding in cln1 cln2 cells (55). It was also isolated as BUDO (56). It plays a role in localization and formation of buds and seems to be the GTPase for Bud1/Rsr1 (55, 56). GTP-bound Rsr1 is thought to interact with Cdc24 (58) and thus ensures polarity establishment at appropriate locations (59). Cdc24 may form a complex with Rsr1-GTP at the presumptive bud site and may thereby get activated in a Cdc28 kinase-dependent manner (53). The formation of this complex might involve Bem1 (60). Cla2 could therefore be a target for Cln2, whose activation would prevent activation of Rsr1. Loss of CLA2 function was found to be synthetic lethal with cln1 cln2 (55). Repression of the mating pathway by Cln2 could involve the activation of bud emergence. For this reason a cla-2 strain was compared with the corresponding wild-type strain, both over-expressing CLN2 from the TPI1 promoter, to see whether Cla2 is necessary for the pathway repression. As it is shown in Fig. 6 (lanes 1–4), a CLA2 deletion has no influence on the down-regulation of the pheromone-dependent gene FUS1 by CLN2.

In summary it can be stated that the putative targets left for Cln2-dependent repression of the mating pathway are Ste11, Ste5, Ste20, and the Cdc42 complex (Fig. 7). Further studies will concentrate on the questions of which of these proteins interact with Cln2, how this interaction occurs, and if Cln2 represses one of the components of the mating pathway directly or indirectly.

**DISCUSSION**

Messenger RNA levels of pheromone-inducible genes fluctuate during the cell cycle (1, 19). They reach their maximum in late mitosis and early G1, drop drastically at START in late G1, and increase again in S phase. In response, pheromone cells have to arrest their cell cycle in G1 before START (2, 3). Cells which have passed START are resistant to mating pheromone (9). Up-regulation of the transcription of pheromone-dependent genes in early G1 may reflect a mechanism that ensures the proper timing of the cell cycle arrest. Expression of the Far1 protein, which is thought to specifically inhibit the G1 cyclins Cln1 and Cln2, is restricted to early G1 (17, 18, 61). After START, Far1 is degraded and the cell is committed to undergo a mitotic cell division cycle (62). Improper expression of a truncated, nondegradable version of Far1 by the GAL1–10 promoter causes cell cycle arrest also in other stages of the cell cycle (budded-cell-arrest phenotype). The cell cycle regulation of Far1 transcription and proteolysis in G1 is one of the mechanisms ensuring the specificity of pheromone arrest in early G1 (62).

The fluctuations observed for transcript numbers of pheromone-dependent genes are oscillating in such a way that they reach their maximum when CLN1 and CLN2 transcripts are down-regulated and drop at the time when CLN1 and CLN2 reach their maximum. Oscillations of the G1 cyclins Cln1 and Cln2 and the accumulation of mRNA of pheromone-dependent genes are counteracting. Oehlen and Cross (1) showed that in fact increased Cln2 levels repress the transcript numbers of pheromone-dependent genes. Cells expressing CLN2 constitutively from the strong GAL1–10 promoter are resistant to pheromone. Cln2-dependent repression of pheromone-induced genes was shown to occur downstream or at the level of the G protein, and does not depend on either SST2 (part of the recovery response, 63) or the NH2 terminus of the pheromone receptor (important for desensitization) (64, 65). We were interested in assessing which components of the mating pathway are affected by a cell cycle-dependent down-regulation and at what level Cln2 represses the pathway.

To address the first question, studies were undertaken to examine the activity of the MAPK Fus3 during the cell cycle. They demonstrate that Fus3 activity is cell cycle-regulated, reflecting the fluctuations in transcript numbers of pheromone-dependent genes. Both basal and pheromone-induced Fus3 activity reach their maximal levels in mitosis and early G1. After START, Fus3 activity is almost absent and increases again during S phase. The cell cycle-dependent regulation of the transcripts of pheromone-induced genes reflects fluctuations in the activity of the MAPK Fus3.

The activity of the MAPK Fus3 is down-regulated in cells overexpressing CLN2 from the induced GAL1–10 promoter, indicating that the pathway and the cell cycle interact at a point upstream of the transcriptional regulation of pheromone-dependent genes. A potential regulator of the observed fluctuations in Fus3 activity seemed to be the Msg5 protein phosphatase. From our experiments the possibility that Msg5 is the mediator of Cln2-dependent repression of Fus3 activity can be excluded.

The whole kinase cascade is cell cycle-regulated in its activity. The activity of Ste7, which lies upstream of Fus3 in the kinase cascade, is subjected to the same cell cycle-dependent regulation as Fus3. Experiments with hyperactive STE11 alleles (51) indicate that Cln2-dependent repression of the mating pathway occurs through Ste11. Induced activities caused by STE11 hyperactive alleles are repressed by increased Cln2 levels. To further analyze at what point Cln2 represses the signal transduction pathway a strain with a deletion in the
RGA1 gene was used (32). RGA1 is thought to act at or before Ste11 in the signaling cascade. Deletion of RGA1 increases the activity of Cdc42. Cdc42 was shown not only to have a role in establishing cell polarity (66) but also in pheromone-dependent induction of the mating pathway (30, 31). A deletion in RGA1 induces the pathway independently of the G protein, but requires the activity of the MAPK cascade. Transcription of pheromone-dependent genes can be observed in ste4 rga1 pbs2 cells, whereby pbs2 enhances the effect of an RGA1 disruption. Overexpression of CLN2 in ste4 rga1 pbs2 cells had no effect on the transcription of pheromone-dependent genes.

There are several conclusions from the experiments with strains harboring the hyperactive STE11 alleles and strains with a deletion in the RGA1 gene: repression of the hyperactive STE11 alleles demonstrates that the site of Cln2 dependent down-regulation has to be downstream or at the level of Ste11. Down-regulation of a STE11–1 allele in a ste11 background indicates that G protein function is not necessary for Cln2-dependent repression of the mating pathway. Experiments with rga1 strains suggest that the repression does not occur downstream of Ste11, because the transcription observed in these strains is not down-regulated by high levels of Cln2. This would be the case if either Fus3 or Ste7 were targets of Cln2. Thus the target of Cln2 seems to be a component involved in Ste11 activity.

Epistasis experiments elucidated the relative positions of components of the mating pathway. It was shown that Ste11 acts upstream of Ste7 and Fus3, as a deletion of STE7, FUS3, or STE12 suppresses the activation of the mating pathway by an hyperactive STE11 allele (51). On the other hand, it was shown that Ste11 acts downstream of Ste3 and Ste4, as a deletion of STE3 (encodes the α-factor receptor) and STE4 (encodes the β subunit of the G protein) had no effect on the capacity of an hyperactive STE11 allele to induce the pathway (51). Ste5 is necessary for the optimal activity of the hyperactive Ste11 kinase and thereby acts at the level or downstream of STE11 (51). Overexpression of STE5 suppresses mutant alleles of pathway components downstream of Ste11. Consistent with these genetic data it was shown that Ste5 interacts with Ste11, Ste7, and Fus3/Kss1 in two hybrid assays. This indicates that it has a role as a scaffold protein, holding together the components of the kinase cascade (21). Rga1 works upstream or at the level of Ste11, as it was shown by the following epistasis experiments: a deletion in STE20, but not in STE5, reduces the signal obtained by an RGA1 deletion (32). A deletion in STE11 abolishes the increased transcription in cells deleted for RGA1. From these data we can think of a linear kinase cascade downstream of Ste11, but at the level of Ste11 different components in parallel may be involved in transmitting the signal from the G protein to the kinase cascade. The hyperactive alleles were not tested in a ste20 background because at this time the Ste20 kinase was not identified yet. The pathway seems to be much more complex at the level of Ste11 than it was initially thought. For this reason it is not possible to exactly define the component of the mating pathway interacting with Cln2.

The different possible interactions between Cln2 and the mating pathway are illustrated in Fig. 7. Cln2 induces budding without pheromone treatment, and morphological changes resulting in shmoo formation are inhibited (18). For this reason experiments determining the role of Bem1 and Cln2 in Cln2-dependent repression of the pathway were done. They were both shown not to be involved in Cln2-dependent repression of the mating pathway.

Further experiments will try to exactly define the point where Cln2 interacts with the mating pathway and how this interaction occurs. We exclude the possibility that the observed repression of the mating pathway is due to a shortened G1 phase and budding at a smaller cell size in cells overexpressing CLN2 since Cross (67) demonstrated that DAF1–1, a mutant allele of the G1 cyclin CLN2, which causes a shorter G1 phase, accumulates FUS1 mRNA to wild-type levels. Cells bud at a smaller cell size, but the intensity of the FUS1 transcript remains the same as in wild-type cells. We expect that overexpression of CLN2 shortens G1, but that this effect is not causing the down-regulation of the gene transcript numbers.

From Fig. 7 it can be seen that the possible targets left are Ste5, Ste20, Ste11, and the Cdc42 complex. Cln2 seems to down-regulate one of these components of the mating pathway either directly or indirectly. On the other hand, it is still possible that Cln2 is repressing several components of the mating pathway concomitantly. It could down-regulate the kinase cascade by repressing Ste11 activity, and promote budding by repressing polarized growth for shmoo formation. The combination of different hyperactive mutants of components involved in the pheromone response will address this issue.

The biological relevance of the studies presented here lies in the demonstration that the cell cycle-dependent regulation of the mating pathway determines its responsiveness and thereby the correct timing of the cell cycle arrest. Also in higher eucaryotic cells the correct timing of responses to external signals has to be in a cell cycle context. Edelmann et al. (68) demonstrated that the activities of the p42mapk/p44mapk also fluctuate during the cell cycle in Swiss mouse 3T3 fibroblasts. It is not known whether upstream components of this pathway underlie the same cell cycle regulation. The studies presented here may be relevant for the proper timing of cell growth and proliferation and may help to elucidate the general mechanism ensuring the correct timing of different responses to external stimuli.

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